THE BIOTECHNOLOGY OF EFFLUENT-GROWN *Spirulina*, AND APPLICATION IN AQUACULTURE NUTRITION

THESIS

Submitted in fulfilment of the requirements
for the degree of

MASTER OF SCIENCE
(BIOTECHNOLOGY)

Rhodes University
Grahamstown

by

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November 1992

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ABSTRACT

The biotechnology of production and utilisation of the cyanobacterium *Spirulina* has been well documented. Research has centred mainly on application in human and animal nutrition, and has been motivated by the high protein, vitamin, fatty acid and growth factor contents. The main obstacle in realising the full potential of this feed source has been the high production costs associated with its mass culture in defined media.

The observation of blooms of *Spirulina* in tannery effluent evaporation ponds in Wellington, South Africa, prompted this investigation into the harvesting, and nutritional and toxicological evaluation of this potentially low-cost production system, with the ultimate aim of using the product in aquaculture rations.

An investigation of the chemical gradient along the evaporation cascade showed a positive correlation between the prevailing chemical conditions and the dominant species populations. A standing crop of 9.5 tonnes/ha of *Spirulina* was found to be present in the latter alkaline ponds, characterised by relatively lower organic and sulphur contents.

Initial harvesting of the biomass was achieved by the design, construction and implementation of a small-scale screen harvest, which yielded a 25 kg (dry weight) crop. A scale-up model was then designed, and implemented in a technical scale harvest, yielding a crop of 250 kg (dry weight). Both these harvests utilised the bloom of surface-autoflocculated biomass. Concentrated cell slurries were sun-dried on muslin beds, and milled to a coarse powder.

An evaluation of the harvest revealed a chemical content similar to other published reports of defined media cultures, with the exception of the protein and amino acid contents. The observed lower levels of the latter two are almost certainly due to the sun-drying method employed, known to reduce the protein content due to thermal denaturation.

Legislation demands the strict toxicological evaluation of new protein sources, and because of the effluent-nature of the growth medium of this source of *Spirulina*, its viability lies only in the application as an animal feed or supplement. A range of toxicological tests were

chosen that were targeted to elucidate the possible toxicological constraints of this effluent-grown source of protein in animal nutrition. The nucleic acid and pesticide contents of the harvested biomass were within the prescribed safety ranges. Atomic absorption showed minimal accumulation of minerals and heavy metals from the effluent. A bioassay with the brine shrimp *Artemia salina* showed that the biomass contained no toxicologically active water-soluble components. A short term feeding trial with new-born chicks showed that supplementation with *Spirulina* had no effect on the growth rates and feed conversion ratios of the different feeding groups. Pathological analyses showed that the liver was the only target organ to elicit a change in response to supplementation of the diets with *Spirulina*. A general decrease in liver weight was noted, with Cu, Ca, Fe and Zn being significantly accumulated. A histopathological examination however, showed no cellular and functional aberration from the control animals.

The toxicological analyses gave the preliminary safe go-ahead for the evaluation of effluent-grown *Spirulina* in aquaculture nutrition. The South African abalone *Haliotis midae*, and the rainbow trout *Oncorhynchus mykiss* were chosen as representative species of edible cultured organisms.

The technology for the culture of the perlemoen abalone is being established in South Africa, with the main area of research being the development of an artificial diet for high density culture. A 40 day growth trial demonstrated that lower concentrations of *Spirulina* supplemented to an agar-based fishmeal diet resulted in growth rates and feed conversion ratios similar to the control fishmeal and purified-casein diets, and thus has application potential in the nutrition of this high-cost marine delicacy.

The aquaculture technology of freshwater rainbow trout is already well established. An eight week feeding trial with various concentrations of *Spirulina* showed that this effluent-grown protein source can partially replace fishmeal in semi-purified diets. Fish fed *Spirulina* did not exhibit decisive manifestations of toxicity, as determined in a histopathological study. In addition, *Spirulina* supplementation resulted in enhanced colouration of the skin and flesh, which may have implications in the aesthetic marketing of this sought-after table fish.

The primary aim of this preliminary investigation thus concerned the determination of the biotechnological potential of this effluent-source of *Spirulina*. A technology transfer from the economically unfeasible defined-media culture was implemented. This project is ultimately aimed as a contribution towards the treatment of tannery wastewater, by the removal of contaminants from the effluent in the form of organic biomass.

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CHAPTER 1

INTRODUCTION

SALINITY AND ALGAL BIOTECHNOLOGY

Summary Industrial wastes present treatment and disposal problems. The field of algal biotechnology has contributed significantly to the amelioration of economic and environmental effects, and forms the initial part of this review. Saline, alkaline tannery effluent has been known to support the growth of the cyanobacterium, *Spirulina*, and the potential of its culture in effluent medium is discussed.

PART A

MICROALGAE AND SALINE WASTEWATER

1.1 INTRODUCTION

Microalgal biotechnology represents a synthesis of fundamental knowledge and rapidly advancing industrial technology. Increasing interest in microalgae can be gauged from the recent appearance of a number of reviews on the applied uses of microalgae (Borowitzka & Borowitzka, 1988a; Cresswell *et al.*, 1989; Richmond, 1986a; Shelef & Soeder, 1980). Research has centred essentially in two fields: The exploitation of microalgae in the extraction of fine chemicals and products of value, and the utilisation of microalgae in wastewater treatment systems, the viability of both these broad-field processes being ensured by the efficient bioenergy conversion ratios exhibited by microalgae (Grobbelaar, 1982). The common element in both these areas of interest is the economic benefits associated with the mass-culture exploitation of microalgae.

Soeder (1986) speculates that the application of microalgae in wastewater treatment appears to bear the greatest potential of all biotechnologies based on microalgae, if exploited fully as

a multi-purpose system. Algal ponds are presently considered to be an economical means for treatment of urban sewage and agricultural wastes (Soeder, 1986). Current wastewater-treatment technologies allow for the harvest and processing of the algal-bacterial biomass from the effluent. Generally, the extraction of various fine chemicals and other economically viable products are hindered by the effluent-source of the growth medium, and the products presently obtained can only be applied as low-value animal protein feed (Richmond, 1986c).

The application of microalgae in wastewater-treatment systems will be reviewed below.

1.2 ORIGIN OF WASTEWATER

Wastewaters can be broadly classified into three main groups:

1. Effluents resulting from municipal activities include stormwater run-off and sewage.
2. Liquid agricultural wastes include effluents from the production of plant and plant products, and animals and animal products.
3. The third group of wastewaters, that of industrial effluents, presents one of the biggest problems for environmentalists. This group encompasses effluents from processing plants and residuals from wastewater management systems (Oswald, 1988a).

Concern has recently centred on the organic and inorganic components of (particularly industrial) wastes, due to the acutely toxic or long-term somatic effects of these components on humans and the surrounding biota. Waste management has become a major concern of governments, and a focus of many pollution laws, rules and regulations to control the emission of wastes. Legislation authorising the expenditure of public and private funds for the collection, treatment, detoxification and disposal of such wastes has become standard practice (Oswald, 1988a).

1.3 WASTEWATER TREATMENT STRATEGIES

Various degrees of treatment are required for wastes from different sources, the type and degree of which depends on the disposal system employed, the available disposal area, the dilution volume, and the potential pathogens and other toxic materials (Oswald, 1988a).

1.3.1 Conventional Treatment Systems

A variety of conventional, non-algal methods are currently employed to attain complete wastewater treatment. These include:

1. sedimentation, flotation and skimming, chlorination, ozonation, UV radiation (for the removal of solids and pathogens),
2. aerated bacterial cultures (for the removal of dissolved organics and pathogens),
3. alkaline caustic treatment (for the removal of plant nutrients and oxidation products),
4. chemical coagulation and activated carbon filtration (for the removal of refractory organics, organic and inorganic toxicants),
5. distillation, reverse osmosis, electrodialysis and solar distillation (for the removal of inorganic salts, residual organics and heavy metals) (Oswald, 1988a).

The relative costs for each of the stages of complete treatment diminishes as the system size increases. The combined costs of treatment and disposal, however, remain relatively high, and the field of environmentally essential wastewater-treatment supports a flourishing industry.

1.3.2 Algae and Freshwater Effluent Treatment

Algal treatment of wastewater involves ponds or impoundments, the most common being facultative and high rate ponds. Facultative ponds are usually more than 1 m deep, have algae growing at the surface while remaining anoxic at the bottom. High-rate ponds are usually less than 1 m deep, continuously mixed by gentle stirring, and are aerobic throughout their volume. The benefits of the use of algae include oxygen production by the algae for waste oxidation by co-existing bacteria, as well as enhancing sedimentation and disinfection,

and nutrient, heavy metal and toxic organic removal (Oswald, 1988a). Different effluent characteristics require adaptations in the design of the system and its operational regime, and are designed for achieving maximal effluent purity at minimal cost (Abeliovich, 1986).

Oswald (1988a) describes a wastewater-treatment system in operation at Hollister, California, as a model system for facultative ponding. The ponds utilised are unmixed, and have algae growing at the surface. Because of the efficient solar energy conversion to heat by the algae, the warm layer at the surface resists mixing, with the lower layers remaining quiescent. The wastewater is injected below the surface of the pond. The still waters ensure near-perfect settling conditions, and a large proportion of the solids settle to the bottom. Due to rapid algal growth at the surface, CO_2 and its derivatives become depleted, the pH increases to as much as 11, and the dominant hydroxyl ions combine with, and precipitate calcium, magnesium and phosphorus. Ammonium, at this high pH, escapes as a gas. The algae become inactivated due to the high temperature and alkalinity, and settle to the bottom. The inactivated algae and settled solids undergo fermentation, and CO_2 and CH_4 are released. Methane fermentation is accompanied by anaerobic denitrification, resulting in the release of nitrogen to the atmosphere. At night, the warm upper layers hasten bacterial degradation, releasing more CO_2 for algal photosynthesis during the day (Oswald, 1988a).

Microalgae have an affinity for polyvalent cations, which offers an advantage in wastewater treatment. Oswald (1988a) recounts a study at Napa, California, of several tanneries emitting chromium compounds. Algae in facultative ponds were shown to adsorb chromium from the water and settle to the bottom, resulting in up to 99% removal of the chromium. Algae autoflocculation is enhanced at high pH's, and polyvalent cations such as chromium, copper, iron, manganese, lead, strontium and zinc are incorporated into flocs, which settle to the bottom in quiescent ponds.

Although the main advantage of facultative ponds is that they require practically no energy input (Abeliovich, 1986). When the pH becomes too high (usually on bright days), bacterial activity is inhibited. This drawback has led to the development of continuously mixed high-rate oxidation ponds (HROP) (Oswald & Golueke, 1960), which ensure continual operation with bacterial oxidation and algal photosynthesis symbiotically complementing each other.

This topic of research has been investigated and reviewed by a number of authors (Abeliovich, 1986; Ganapati, 1975; Oswald,1988a).

If all factors are maintained at optimum levels, algal growth rates in HROPs can be maintained at doubling times of 3-4 days for very long periods of time (Abeliovich, 1986). Algae have to compete with bacteria on available nutrients, thus only those algae capable of heterotrophic growth survive and predominate (Abeliovich & Weissman, 1978).

The final polished effluent is usually of sufficiently high quality to be released into the public water system. In addition to this, an algal-bacterial biomass is obtained, which has application as a protein supplement in animal feeds (Soeder, 1986).

1.4 SALINE WASTEWATERS

1.4.1 Origin of Saline Wastewaters

The problem of rising salinity levels in South Africa's freshwater supply has been identified by Allanson *et al.* (1990) and Rose (1992). Environmental evaluations of the Department of Water Affairs (DWA) conclude that salinity is the single most serious threat of pollution facing the public water system in South Africa (Stander, 1987).

Salinity is one of the most intractable forms of pollution, and may arise from one of three sources:

1. Geological salinity originates when elevated water tables give rise to highly saline base flows, the effect being intensified by evaporative losses caused by elevated temperatures and drought, an environmental characteristic of South African climate (Allanson *et al.*, 1990).

2. Agricultural salinity arises due to the accelerated release of natural salts from soils, which are then released into the water system. This problem arises especially during dry land farming. Continuous irrigation, in conjunction with evapo-transpiration,

results in an accumulation of salt in the upper layers of the soil, with serious agricultural consequences (Rose, 1992).

3. The third cause of salinization, which forms the main incentive for this review, is that of salinity arising from industrial activity, where rising salt levels are not only a threat to downstream agricultural land, but also add an unacceptable cost to domestic and industrial users (Rose, 1992).

1.4.2 Industrial Saline Effluent

Industries produce a large proportion of South Africa's saline wastewater, with tanneries having been described as producing the most polluted waste of any industrial activity (Tsotsos, 1986). The main problem associated with tannery wastewater is the high NaCl concentrations which are used for salt curing of hides. Because of the pollutant potential of this effluent, various techniques have been proposed and investigated. Desalination may present a solution, although, like all other wastewater treatment systems, this does not eliminate effluent, but converts it into a more manageable form, in terms of volume reduction. Desalination has been most efficiently achieved through the use of reverse osmosis (RO), where 80-90% of the saline water is converted into pure water. Under optimum conditions, the industry is still left with 10-20% of the effluent, now a very concentrated brine solution, which still has to be disposed of (Cooper *et al.*, 1983; 1984).

Before RO desalination can be implemented in saline wastewater treatment, it is necessary to subject the effluent to preclarification procedures necessary to remove soluble and insoluble matter which would otherwise result in membrane fouling. Preclarification can be achieved through ultrafiltration or cross-flow filtration (Cooper *et al.*, 1984). However, the cost implications of this degree of technology is severly limiting (Neytzell-de Wilde *et al.*, 1987).

The treatment of tannery wastewater to meet environmental discharge standards requires the construction of expensive effluent treatment systems, and the implementation of membrane desalination techniques. In addition to capital and maintenance costs, the municipal discharge

rates have to be accounted for. Rowswell *et al.* (1984) have therefore proposed the building and implementation of evaporation ponds which would involve minimal costs for effluent treatment due to effluent volume reduction and the low cost technology involved. Such a system is presently in operation at Western Tanning Company (WTC), Wellington, South Africa (Rose, 1992), and is a spontaneous occurrence of the facultative pond previously described, with an algal-bacterial symbiosis in operation.

The main disadvantage of this ponding system is that valuable water is lost through evaporation. Also, a concentrated brine solution is left at the end of the evaporation process, which still presents a disposal problem (Rowswell *et al.*, 1984). Studies of RO desalination for the DWA estimate the cost of brine disposal at 10% of the total cost of the plant. This figure escalates several fold if plastic pond lining is used to prevent ground water contamination, as in the system employed at WTC, Wellington (Rose, 1992).

Rose (1992) comments that effective technology for brine disposal and associated costs are major limitations in developing a long-term strategy for the treatment of saline wastes. Because of the high cost of the treatment processes, a solution is the beneficiation of the saline effluent, in order to transform a problem into a valuable resource. One way to overcome the already high, continually escalating costs of desalination is to recover products of value from the effluent, a concept already well established in existing biotechnological processes (Hacking, 1986).

1.4.3 Algae and Saline Wastewater

The broad aim of this research programme is to attempt to compensate, in part, at least, for the high cost of tannery effluent treatment, by the feasible incorporation of an algal-technology system. Existing technology for the utilisation of algal processes and products is centred in two main fields: that of wastewater treatment discussed above, and also the recovery of products and/or biomass of value.

Saline brines are unique in their salinity, and, as such, afford a selective advantage to organisms exhibiting halophilic phenotypes. The utilisation of the halotolerant algae

Dunaliella (Borowitzka, 1988) and the cyanobacteria *Spirulina* spp. (Richmond, 1988) in mass-culture is well documented.

Dunaliella spp. are mass-cultured as a food source in aquaculture (Spectorova *et al.*, 1982), as a commercial source of β-carotene (Ben-Amotz & Avron, 1983) and, to a lesser extent, as a source of natural glycerol (Chen & Chi, 1981). *D.salina* is the first microalgae to be used commercially to produce fine chemical as its extreme salinity tolerance simplifies maintenance of a mono-algal culture, relatively free of competitors, pathogens and predators (Borowitzka & Borowitzka, 1988b). Recently, the feasibility of cultivating *D.salina* in saline effluent as an alternative media source has been demonstrated (Rose & Cowan, 1991a), as well as the use of this alga in the organic load reduction of tannery effluent (Rose & Cowan, 1991b).

The cyanobacterium *Spirulina* has been utilised for decades as a protein source by the natives of Chad and Mexico (Ciferri, 1983). The high protein, vitamin and mineral contents of this cyanobacterium make it suitable for both human (Becker, 1986) and animal nutrition (Richmond, 1988; de Pauw & Persoone, 1988). In addition to the nutritional advantages associated with the use of *Spirulina*, mass culture-of this cyanobacterium also yields a number of commercially significant by-products, in the form of the accessory pigment phycocyanin, and the essential fatty acid linolenic acid (Richmond, 1986b).

Species of *Spirulina* have been found in a variety of environments: soil, sand, marshes, brackish water, seawater, freshwater, thermal springs, warm waters from power plants, fish ponds, etc. Of particular interest here is the observation that certain strains of *Spirulina* inhabit salt pans (Ciferri, 1983). This organism therefore seems capable of colonising environments in which life for other organisms is, if not impossible, very difficult. Moreover, reports have also been made of *Spirulina* occurrence in alkaline lakes of Mexico and Africa. In addition, many the alkaline lakes of the Chad region in Africa have high salt (carbonates and bicarbonates) concentrations, and are characterised by virtual mono-cultures of *Spirulina* (Itis, 1975, cited in Ciferri, 1983).

Commercial production strategies for the exploitation of *Spirulina* have already been established in Mexico, Taiwan, USA, Thailand, Japan, and Israel, with a world production of 850 tonnes in 1984 (Richmond, 1986b). Existing production strategies employ controlled, freshwater cultivation.

Spirulina has also been cultivated in effluent medium, at best only on an experimental scale. Organic waste from cattle feedlots supplemented with NaCl has successfully been used in the cultivation of this cyanobacterium (Mitchell & Richmond, 1987; Mitchell & Richmond, 1988). A mono-culture was maintained by the introduction of the rotifer *Brachionus plicatilis*, who selectively scavenged the unicellular contaminants.

It has been noted by Rose (1992) that the WTC tannery evaporation ponds in Wellington support a rich growth of *Dunaliella* and *Spirulina* spp. The cultivation of *Dunaliella* in this media has already been reported by Laubscher *et al.*, (1990), who demonstrated the economic feasibility of the exploitation of this resource.

The occurrence of *Spirulina* in tannery effluent evaporation ponds represents what would usually be considered a pilot-scale production procedure, by virtue of the production scale and volume. It seems that subsequent investigations on the potential use of this source of *Spirulina* would represent a scale-down procedure, as outlined by Trilli (1986). This would not only provide valuable information on the potential application of this cyanobacterium, but also a biological optimisation and cost analysis of the cultivation process could lead to significant production cost saving and increase in productivity.

PART B

THE BIOTECHNOLOGY OF *Spirulina* PRODUCTION

1.5 INTRODUCTION

Spirulina is a multicellular, filamentous cyanobacterium. The filaments are helical, unbranched and motile, and nitrogen-fixing heterocysts are absent. This species of cyanobacterium (blue-green alga) has been reported to reach very high densities in waters capable of supporting it's growth. Rich in 1931 made one of the first reports of the abundance of *Spirulina* in the alkaline, saline lakes of Africa and the Americas (Richmond, 1988).

Cyanobacteria are unique in that, although their procaryotic cellular organisation is similar to that of bacteria, they are generally obligate photoautotrophs, obtaining their carbon and energy from photosynthetic mechanisms which are similar to those found in higher plants (Holm-Hansen, 1968). It has, however, been established that cyanobacteria also possess mechanisms that enable them to grow heterotrophically in the dark, utilising a variety of organic compounds (Diakoff & Scheibe, 1975; Hoare *et al.*, 1967; Rippka, 1972; Smith *et al.*, 1967; van Baalen *et al.*, 1971)

Nevertheless, it is because of a predominantly photoautotrophic nutritional mode that these organisms were first classified by botanists. Stanier, *et al.* (1978) were amongst the first to suggest that cyanobacteria be placed under the rules of the International Code of Nomenclature of Bacteria. This proposal was later unanimously accepted by the International Association of Microbiological Societies (IAMS) (Stanier, 1977), ruling out the previous unsuccessful attempts at classification by the Botanical Code classification, as noted by Golubic (1979).

Although the helical shape of the *Spirulina* trichome is characteristic of the genus, taxonomic characterisation of the Oscillatoriaceae has presented a problem, due to the array of

morphological and anatomical variants found in response to variation in physical and environmental conditions (van Eykelenberg, 1979, 1980). Nevertheless, taxonomic classification to the genus level has leaned heavily on helix characteristics. Tentative species classification has also relied almost entirely on helix structure (Richmond, 1988).

Vonshak (1987) has developed a protocol for the isolation and comparison of the photosynthetic membrane fractions on poly-acrylamide gradient gels. Although few distinguishing differences between the strains could be noted, this approach is being developed further by the use of other protein fractions.

Fatty acid distribution has also been used in the taxonomic classification of *Spirulina*, and although it has been found useful to the level of class and order, identification to the genus level is not possible (Kenyon *et al.*, 1972). Cohen & Vonshak (1991) however, found that cyanobacteria morphologically indistinguishable from *Spirulina* exhibited patterns of fatty acid distribution which are different from *Spirulina* both quantitatively and qualitatively. They therefore proposed that the fatty acid composition could be used as an additional criterion for the characterisation of the genus, along with the already existing morphological criteria.

Based on trichome morphology these cyanobacteria have been grouped into two major species:

1. *Spirulina platensis* (synonyms *S.jenneri* var. *platensis* , and *Arthrosphira platensis*)

2. *Spirulina geitleri* (synonyms *S.maxima*, *S.platensis*, *A.maxima*, and *Oscillatoria platensis*)

Due to the uncertainty of the taxonomic classification, morphological variation and the large number of synonyms used for the same species, the term *Spirulina* will be used here at a genus level, unless direct reference is made to a species.

1.6 ECOLOGY

Spirulina is a ubiquitous organism, and has been found in soils, marshes, fresh, brackish and sea waters, and thermal springs, colonising extreme environments that are unsuitable for other organisms. This wide distribution is a consequence of the ability to use atmospheric nitrogen as the sole nitrogen source. This means that *Spirulina* requires for growth only two gases; CO_2 and nitrogen, H_2O as an electron donor, and a number of inorganic elements, the most important of which is phosphorus. Some aquatic forms can survive dessication for long periods of time. Soil-dwelling forms are important in that they contribute to soil fertility through their ability to fix nitrogen (Gaudy & Gaudy, 1980).

Cyanobacteria often appear in vast numbers as blooms in waters that contain sufficient concentrations of the required nutrients and minerals (Gaudy & Gaudy, 1980). Typical examples are *S.maxima* and *S.platensis* which bloom profusely in certain alkaline lakes in Central America and Africa (Richmond, 1988). Blooms are characterised by floating mats of the cyanobacterium that result from autoflocculation. This occurs because of the presence of nitrogen-containing gas vacuoles enabling them to float at the surface, or at a depth at which light intensity is optimum for photosynthesis (Gaudy & Gaudy, 1980).

Iltis in 1974 concluded from his studies on the populations characterising alkaline waters, that lakes with a salt concentration below 2.5g/l support the growth of chlorophytes, cyanophytes and bacillariophytes (Richmond, 1988). In mesohaline lakes (salt concentration 2.5-30.0g/l) cyanobacterial populations dominate, comprising *Synechrocystis*, *Oscillatoria*, *Spirulina*, and *Anabaenopsis* spp. In alkaline lakes where the salt concentration is over 30.0g/l, *Spirulina* can be the only organism present. This phenomenon is found in Lake Chad, where the alkalinity ranges from pH 7.2-9.0, the dominant ion being HCO_3^-. The inhabitants of Chad have been using *Spirulina* as a source of protein for centuries.

Thus, *Spirulina* predominates at higher pH values and salt concentrations.

Ciferri (1983) found that a similar situation existed in Lake Aranguadi (the "Green Lake") in the Rift Valley in East Africa, where the pH reached 10.3 at certain times of the year.

He found that *Spirulina* was the only micro-organism present, and was entirely responsible for the high photosynthetic rate observed. This substantiates a study by Ogawa & Aiba (1978), who established that *Spirulina* possessed a relatively high CO_2 fixation rate, with negligible photorespiration.

Murphy, *et al.* (1976) found that the dominance of cyanobacteria can also be entirely or partially ascribed to an iron deficiency in the medium. During iron-deficient conditions, cyanobacteria excrete trihydroxamates (catechols) which can selectively chelate ferric iron. Neilands (1967) postulates that these low-molecular weight chelators act as carrier molecules, transporting iron across membranes. Species with enhanced iron uptake systems like those possessed by cyanobacteria would thus have a selective advantage in this ecological niche. These hydoxamate chelators have also been found to inhibit the growth of other algae (Murphy, *et al.*, 1976).

Spirulina is a thermophilic cyanobacterium, with the optimal temperature for growth being 35°-37°C. The minimum temperature that still supports growth is 18°C (Richmond, 1986). It is because of this thermotolerence that cyanobacteria are the major photosynthetic agents in deserts, and almost the exclusive dominant photosynthetic population in thermal springs (Stanier, 1977). Tomaselli *et al.* (1988) found that increasing temperatures of *S.platensis* M2 cultures resulted in a concomitant rise in carbohydrates and lipids, and a decrease in specific growth rate, chlorophyll a, phycobiliproteins and total protein.

1.7 *Spirulina* AS A FEED SUPPLEMENT FOR HUMANS

Microalgae, with their rapid growth rates and utilisation of renewable resources (Borowitzka & Borowitzka, 1988) are efficient producers of high-protein biomass. *Spirulina*, because of its high available protein and vitamin content, relative ease of exploitation, and long history of human consumption (Ciferri, 1983) is a favoured organism for this application (Fox, 1987).

Where proper conditions for growing *Spirulina* can be achieved, there is little difficulty in culturing of this organism. However, in spite of protein productivity an order of magnitude

greater than conventional sources, the greatest problem has been motivating the finance and approval of a serious world-wide effort to exploit *Spirulina* as a means to end malnutrition (Fox, 1987).

The first report on the use of *Spirulina* as a staple feed in human nutrition was made in 1524 by Fray Toribio de Benavente, who noted the harvest of "tecuitlatl" (floating mats of *Spirulina*) from lakes in Mexico. The natives would harvest the cyanobacterium from the water using a fine mesh, sun-dry the biomass, and use it as a staple food, or as a supplement to their diet (Richmond, 1986b).

Identification of the organism came later, however, with the discovery of the "dihe" cake eaten by the Kanembu people along the shores of Lake Chad in central West Africa. Prevailing winds push the cyanobacterial mats towards the shore, where it becomes concentrated into a thick mash. The biomass is then sun-dried in sand depressions. Final drying is achieved on mats, and the dihe then cut into small, brittle cakes, cooked into a sauce, and eaten with millet as a staple diet (Richmond, 1986b). It is noted by Ciferri (1983) that, depending on the season, dihe is eaten in seven of ten meals. Pregnant women in this tribe eat the dihe cakes as a superstitious protection of the foetus. The dark colour of the cake supposedly screens the baby from the eyes of sorcerers.

Ciferri (1983) also recounts tests performed by the Mexicans, who showed that *Spirulina* has nearly the high quality of whole egg protein, and contains a rich source of vitamins and minerals. As a consequence, *Spirulina* biomass is produced by the Mexicans on a commercial scale in the Lake Texcoco region.

There are numerous factors pointing towards the advantageous use of *Spirulina* in human nutrition. It has a relatively low percentage of nucleic acids (4%) as compared to the higher concentrations in other single-cell protein sources. The mucoprotein cell walls are easy to digest as opposed to the cellulose cell wall found in other microalgae. Pure-culture-grown *Spirulina* is completely non-toxic, and does not produce any of the toxins characteristic of other species of cyanobacteria. The lipids are made up of unsaturated fatty acids, and do not form cholesterol (Richmond, 1988).

Spirulina has a high protein and vitamin content. 20g Dried *Spirulina* provides the full RDA of vitamin B_{12}, 70% RDA for thiamine, 50% RDA for riboflavin, and 12% for niacin. Other favourable nutritional attributes of *Spirulina* include the high content of β-carotene (pro-vitamin A) and essential unsaturated fatty acids (Richmond, 1988).

Aside from the nutritional properties of *Spirulina*, reports have also been made on the therapeutic properties associated with this cyanobacterium:

1. Pharmaceutical compounds containing *Spirulina* as the active ingredient induce accelerated cicatrization of wounds.

2. *Spirulina* and it's enzymatic hydrolysates promote skin metabolism, and prevents the formation of scar tissue.

3. Iodine present in *Spirulina* is the same type as that found in thyroid glands, and feeding with *Spirulina* has been found to result in growth stimulation.

4. Phycocyanin is an accessory blue pigment in *Spirulina* (Boussiba & Richmond, 1980), the concentration of which is controlled by the prevailing lighting conditions (Bryant, 1981). When given orally to laboratory mice, phycocyanin resulted in a significant decrease in death when the mice were exposed to liver tumour cells. It is thought that phycocyanin generally stimulates the immune system, which may explain the higher lymphocyte activity found in the experimental group (Richmond, 1988).

5. The intake of β-carotene (provitamin A) has been linked to a reduction in cancer risks. The high β-carotene level in *Spirulina* suggests that it to may decrease certain cancer risks when ingested in appropriate amounts (Richmond, 1988).

6. *Spirulina* is a concentrated source of gamma-linolenic acid (GLA) (Nichols & Wood, 1968) which is a precursor of prostaglandin E_1 (PGE). PGE is involved in many essential tasks in the body, including the regulation of blood pressure, cholesterol synthesis, inflammation and cell proliferation. Studies have shown that GLA (and

subsequently PGE) may aid in the combatting of arthritis, heart disease, obesity and zinc deficiency. GLA deficiency has also been linked to alcoholism, manic depression, aging symptoms and schizophrenia (Richmond, 1986b). Cohen *et al.* (1987) found that the concentration of GLA and other fatty acids could be manipulated by changing the environmental conditions. Most significantly, an increase in cultivation temperature leads to an increase in the fatty acid content and a relative decrease in the amounts of polyunsaturated fatty acids.

7. *Spirulina* extracts restore cholinesterase activity in human erythrocytes that had been inhibited by organo-phosphate pesticides (Richmond, 1986b).

The most viable use for *Spirulina*, however, seems to lie in its nutritional properties. Regulatory procedures for the evaluation of new food products are rigorous, and of the same magnitude and intensity as the testing of novel pharmaceutical compounds (Hacking, 1986). The toxicological considerations, in conjunction with the resistance of humans to novel sources of protein, has led to the development of an algal animal-feed industry. The passage of algal protein through an animal source thus forms the basis of a double-industry, where the final product forms a more conventional component of the human diet.

1.8 *Spirulina* FOR AQUACULTURE

Aquaculture is one of the most rapidly growing fields for food production. The last decade has seen nearly a tenfold increase in production, with more than ten million tonnes currently being produced (de Pauw & Persoone, 1988).

The utilisation of microalgae in the diet of aquaculture species has been investigated by a number of authors (Ahmad, 1966; Gupta & Ahmad, 1966; Matty & Smith, 1978; Reed *et al.*, 1974; Sandbank & Hepher, 1980; Stanley & Jones, 1976).

Microalgae are the biological starting point in the aquatic grazing food chain, so it is logical that they are incorporated in the commercial production of certain aquatic organisms. Aquaculturalists rely on live microalgae as feed for commercially important species for at

least the larval stages of development. Animals that require microalgae for larval development include marine bivalve molluscs (oysters, scallops, clams and mussels), the larvae of some marine gastropods (abalone), larvae of saltwater shrimp (*Penaeus* and *Metapenaeus*), some fish species (*Tilapia*, silvercarp), and zooplankton. The zooplankton, in turn, serve as feed for a range of freshwater and marine fish and crustaceans. Zooplankters that are commonly used include rotifers (*Brachionus*), copepods (*Tigriopus*), cladocerans (*Daphnia, Moina*) and brine shrimp (*Artemia*). (de Pauw & Persoone, 1988).

A semi-commercial design in Cape Cod, USA utilises three separate and relatively pure algal cultures in unheated water for the feeding of artificially reared clams. The three species used allows for compensation for change in seasonal growing conditions. The algae is then diluted with seawater and pumped through the hatchery beds, where the clams extract the protein source by filter feeding (Ehrenberg, 1980).

It has also been found that algae may add a growth factor to the culture medium of the larvae, enhancing survival and growth (Cohen, *et al.*, 1976).

Experiments by Stanley & Jones (1976) revealed that *S.platensis* is an adequate protein source for the cultivation of bigmouth buffalo fish (*Ictiobus cyprinellus*) and blue tilapia (*Tilapia aurea*), even as a single-ingredient feed. The feed conversion, which is a ratio of feed consumed to unit weight gained, is used as an indicator of the efficiency of the feed. Feed conversions of up to 2.0 have been recorded by Stanley & Jones (1976), which is excellent for a single-ingredient feed. Results indicate that aquaculture with *S.platensis* as a protein source is feasible, and conversions of 2:1 are acceptable if the feed consists of an inexpensive ingredient. However, the alga used in their study was grown at considerable expense, and they theorise that natural populations of alga could be harvested at less cost, making the production system economically viable. Stanley & Jones (1976) also recommend that an improvement in protein conversion efficiency may be achieved by supplementing the diet with a low-cost carbohydrate feed.

Ehrenberg (1980) also reports a growth increase of 62.5% and 100%, respectively, when feeding pure *Spirulina* to *Tilapia rendalii* and *Cyprinus carpio specularis*. Researchers at the

Fish Farming Experimental Station in Arkansas obtained similar results. They speculate that the greatly increased growth in algae-fed fish cannot be accounted for by either the quality or quantity of protein added. Thus, it seems that all feed is converted more efficiently in the presence of algae.

Earthrise Farms, a commercial producer of *Spirulina* in California, estimate that Japanese fish farmers used about $2.5 million worth of *Spirulina* in 1989 (Henson, 1990). Their promotional literature lists the key benefits associated with the use of *Spirulina* in aquaculture:

1. Better growth rates are obtained, and less feed is wasted because of the inherent palatability of *Spirulina*. Fish fed with this cyanobacterium have less abdominal fat, the energy being redirected into growth. This hypothesis was tested and verified in feeding trials with Cherry salmon.

2. Fish fed *Spirulina* have an improved quality in terms of flesh flavour, consistency and colour. Henson (1990) reports a study by Matsuno (1979), where Sea Bream, Mackeral, Yellowtail, and ornamental koi carp exhibited enhanced colouration upon feeding with *Spirulina* supplements.

3. Henson (1990) also reports a study by Kato (1988) where yellowtail exhibited enhanced survival rates after being reared with *Spirulina*, mortality rates dropping by 14%.

4. The reduced toxicity and increased effectivity of fish medications in fish fed *Spirulina* has been attributed to the blue pigment, phycocyanin. Henson (1990) quotes conclusions of a study by Yamane (1988) where *Spirulina* reduced the toxic effects of heavy metal contamination in certain fish.

At present, *Spirulina* is utilised mainly in the concentrated and preserved state. Spray-dried *Spirulina* has been used in the production of *Artemia* , sun-dried *Spirulina* in the rearing of *Tilapia* and spray-dried *Spirulina* for *Brachionus* (de Pauw & Persoone, 1988).

Matty & Smith (1978) used *Spirulina* in a comparative study with other single-celled protein sources in the feeding of rainbow trout. They obtained favourable growth rates at high inclusion percentages of the cyanobacterium, although the maximum protein efficiency ratios were obtained with the bacterial and yeast biomass also tested.

One of the major areas of research into the aquacultural significance of *Spirulina* has been the colour enhancement potential, due to the unpopularity of artificial dyes (Hardy *et al.*, 1990; Gall, 1992). *Spirulina* has been used in the colour enhancement of ornamental koi carp, trout, salmon, shrimp (Ehrenberg, 1980), sweet smelt (Mori *et al.*, 1987; Henson, 1990) red tilapia (Boonyaratpalin & Unprasert, 1989) and the striped jack (Okada *et al.*, 1991).

Use of *Spirulina* as a protein and pigment source in aquaculture has been hindered by the high production costs of pure-culture grown biomass. As a consequence, the algae has either been used as a starter feed only for larvae, or as a specialist feed (e.g. for colour enhancement in ornamental fish). Considering its nutritionally complete nature of *Spirulina*, it seems that this cyanobacterium would provide a novel feed source for aquaculture organisms if the production costs could be reduced to a minimum.

1.9 MASS-CULTIVATION OF *Spirulina*

The first practical consideration at the onset of an algal mass-production system is that of pond design. A compromise must be found between the financial input and the expected returns.

In general, the simplest and most cost-effective systems for the mass culture of microalgae are shallow raceways or ponds, agitated by paddle-wheels. Such systems, although simple in appearance and operation, are the result of detailed engineering design to optimise operation. This includes optimised mixing to prevent settling, facilitate light transfer and maximise productivity. Typical problems associated with these systems are contamination

by other algae, lack of temperature control, and low cell densities and/or productivity due to light limitation (Anderson, 1985).

Nevertheless, open ponds are still the most popular production system, and are either open, or covered with a transparent material (Richmond, 1988). Top-covered ponds are essential for the maintenance of the relatively high temperatures required for the optimum production of *Spirulina*. Covers trap longwave radiation during the day, prevent radiative cooling at night and reduce evaporative losses. Evaporative losses increase the cost of water and pumping, and it also increases the salinity of the medium, resulting in retarded algal growth. This necessitates medium replacement, which substantially increases production costs. A closed system also reduces the dirt and insect contamination of the final product. This system has been used for mass-scale *Spirulina* production at the Institute for Desert Research, Sede Boqer, Israel (Richmond, 1988).

An alternate design described by Ciferri (1983) uses closed polyethylene tubes as reactors for the production of *Spirulina*. The tubes are arranged on the ground in a raceway fashion, and the medium is circulated by a pump. Bocci *et.al.* (1988) used glass photobioreactors in their studies on the growth physiology of *S.platensis* under natural light using ammonia or nitrate as a nitrogen source. Although impractical for large-scale mass cultivation, their experiments have reinforced the observations by van Rijn & Shilo (1986) that high light intensity results in more fixed carbon being incorporated into polysaccharides than into protein. During periods of low light and at night, the synthesised polysaccharides are used for protein synthesis. This implies an adaptation developed by phototrophic microorganisms to the circadian rhythm.

Because of the advanced design features incorporated into completely-closed systems, the problems typically associated with mass culture in open ponds can be avoided. The closed vessel design and positive head pressure can eliminate undesired contamination. Temperature and other operational parameters can be controlled precisely in a range optimal for growth and product formation. Culture depth can be operated at optimum levels to avoid light limitation and allow for higher cell densities. Gas sparging systems employed in closed systems provide excellent mixing and efficient addition of CO_2 (Anderson, 1985). Volumes

associated with closed systems (e.g. a tubular reactor) are low, comprising one fourth to one-tenth of that typical of channel raceways. The small volumes facilitate the maintenance of population densities several-fold higher than those optimal for conventional channel raceways. The high densities, in turn, reduce the cost of the medium and increase the harvesting efficiency. Closed systems prove useful in elevating the temperatures, a major limitation for algal growth during the winter months. The advantages of a closed system are thus generally those of provision of optimal conditions for growth and constantly high productivities (Anderson, 1985).

Closed systems also have some disadvantages. Light penetration is restricted, as most of the commercially available materials are not completely transparent. Dust accumulates on the outside, and water condenses on the inside, reducing the radiation by up to 40%. Closed systems also increase the capital outlay by 15% (Richmond, 1988), which is the main reason why open ponds are the preferred production system.

It is obviously advantageous to maintain a maximum biomass output rate, and this occurs at a relatively high population density, where the growth rate is approximately 50% of the maximum (Richmond, 1988). It has been found that mixing is the most important factor affecting population density. Turbulent flow results in maximal production, as it ensures that a favourable regime of light intermittence is maintained (Richmond & Vonshak, 1978). Laminar flow results in low efficiency of solar energy utilization because at a given time interval, most of the culture is exposed to insufficient irradiation, while the algae at the surface layer is exposed to radiation intensity which, at best, cannot be efficiently utilised, and, at worst, causes damage through photoinhibition and photooxidation. Turbulance is thus needed to ensure that boundary layers do not form in terms of nutrients, gases and light (Richmond & Vonshak, 1986). Again, the disadvantage of installing a paddle-stirring device is that it increases the investment and maintenance costs of the culture (Richmond, 1988). Cardenas & Markovits (1985) have noted that paddle wheels consume 30-50% of the usable energy in the harvested yield.

In Japan, Dainippon Ink & Chemicals Inc. (DIC), have developed a cultivation pond for the mass cultivation of *Spirulina*, consisting of an oblong open channel based on a circulation

system that is provided with paddle wheels generating a uniform flow. System design has ensured that neither partial flow nor stagnation occurs at any part of the pond, resulting in significantly increased output rates. This production system has been patented in Japan and the USA (Shimamatsu & Tominaga, 1980, cited in Shimamatsu, 1987). DIC presently has two factories for mass production of *Spirulina* in Bangkok and California. Accumulated evidence shows that the DIC ponds are of the highest efficiency in terms of biomass output (Shimamatsu, 1987).

1.10 HARVESTING AND PROCESSING OF *Spirulina*

Harvesting of microalgae from the growth medium has presented one of the technological bottlenecks in the commercial production of microalgae. This constraint is not as severe in the harvesting of *Spirulina*, as the filaments are long enough to be removed from the medium by simple filtration. Sieve nets, vibrating screens and microstrains have been used for *Spirulina*, with limited success. All the above methods result in either the loss of some of the biomass into the return flow, or cell breakage. Cell breakage results in organic enrichment, which encourages the growth of contaminants. Because the meshes used for the harvesting of *Spirulina* have relatively large pore sizes, it allows for the concentration of the prevailing contaminants. Harvesting of the biomass should thus ideally meet the two criteria of total biomass removal, with no cell breakage. To date, such a system has not yet been developed (Richmond, 1988).

Drying of the *Spirulina* biomass may constitute up to 30% of the production costs. Different drying systems alter both the food value and taste of the product. The three most widely used methods for drying of the biomass are spray-, drum-, and sun-drying. Spray- and drum-drying yield products which are suitable for human consumption. The sun-dried product, however, is not usually suitable for human consumption as the process is slow, resulting in an unpleasant odour, degradation of some of the biomass, and a higher bacterial count. Sun-drying is, however, acceptable for the production of animal feed (Richmond, 1988).

1.11 COMMERCIAL PRODUCTION OF *Spirulina*

The main impediment of large-scale mass-production of *Spirulina* is the high production costs. 40% Of the production costs stem from capital costs. Emphasis is therefore placed on maximal yield, which can amount to over 30 g/m²/day. This, however, is a theoretical figure, and has not been practically realised.

In 1984 it was estimated that a plant producing 15-25 tonnes annually of spray-dried *Spirulina* would necessitate an overall capital investment of 6-8 million $(US) per tonne. In 1984, the wholesale price of *Spirulina* ranged between $ 10 000-20 000/ tonne, depending on the quality of the product. It seems then that the financial returns on *Spirulina* production could be lucrative if the plant is run at maximal production for a long enough time (Richmond, 1988).

At present, the commercial production of *Spirulina* occurs in Mexico, Taiwan, Thailand, California, Japan and Israel. The different prevailing conditions dictate the type of production system, and the choice of materials for pond construction and bacterial nutrition.

In Mexico, the largest production plant is in Lake Texcoco, in the Valley of Mexico, where naturally occurring populations of *S.maxima* are harvested by filtration, homogenized, pasteurized and spray dried. Santillan (1982) reports that daily production of the plant approaches 2 tonnes dry weight, with an annual production of 28 tonnes protein/ha. This source is marketed as a specialist health food, and as a feed source for Japanese ornamental fish to enhance skin colour (Ciferri, 1983). Proteus Inc. is the distributer of *Spirulina* from Lake Texcoco. Approximately 500 tonnes/year are exported to Japan, and the remainder to Canada, the United States and Europe (Ehrenberg, 1980).

Commercial cultivation in Brazil is restricted to two private industrial companies with a production capacity in 1987 of 1 tonne per month and a productivity of 6-15 g dry weight/m²/day. Ponds are of 500-2000 m², and are agitated with paddle wheels. It is envisaged that mixing boards will eventually replace the paddle wheels, because of the

advantages of more effective vertical mixing and lower energy consumption (Wagener & de Luca Rebello, 1987).

Spirulina production in Chile comprises the use of 30 shallow cultivation ponds agitated by a drag board. The medium used is made of 30% sea water and fresh water from the Huasco River, to which various minerals are added. Medium is recirculated after being purified in an adsorption charcoal filter and carbonated with pressurised CO_2 (Valderrama *et al.*, 1987).

Production of *Spirulina* in Italy has also centred on the use of seawater as a medium. A procedure for the cultivation in untreated, fertilised seawater was developed. The use of seawater is recommended for at least two reasons. Firstly, the regions of the world with favourable climatic conditions for the cultivation of *Spirulina* are generally also characterised by an acute deficiency of freshwater, while seawater is available in unlimited amounts along coastal areas. The use of seawater also reduces the consumption of chemicals for the formulation of the culture medium. Use of this cultivation system has resulted in production of 7.3 $g/m^2/day$ in seawater supplemented with urea, and 5.2 $g/m^2/day$ in sea water supplemented with nitrate (Tomaselli *et al.*, 1987).

In Thailand, the Siam Algae Company produced 100 tonnes/annum (Richmond, 1986b).

In the United States, Earthrise Farms in the Imperial Valley in California were the pioneers of *Spirulina* production. They operate a variety of ponds of different capacities, as well as an array of smaller experimental ponds (Earthrise, California; Richmond, 1986b). As in Mexico, Proteus Inc. is also the distributer for this source of *Spirulina*. The Proteus system uses unheated water which permits an 8-9 month growing season. It is estimated that chemical costs are of the same magnitude as production costs. In addition to this, high capital costs mean that the minimum cost-effective size is estimated at 25 acres (Ehrenberg, 1980).

In Israel, two commercial plants are into full-scale production, producing each, on average, 10-20 tonnes/annum.

Table 1 summarises the world production of *Spirulina*.

Table 1 Commercial production of *Spirulina* for food (1984) (after Richmond, 1986b)

Country	Number of Production Plants	Area (ha)	Annual Production
Mexico	1	10	300
Taiwan	4	16	300
U.S.	1	5	90
Thailand	1	1.8	60
Japan	1	1.3	40
Israel	2	1.5	30
World	10	35.6	850

Of prime importance in the economic viability of algal culture is the maintenance of maximal output rates. Different approaches have been used in the pursuit of the optimization goal. Historically, the first of these was a trial and error approach, in which the best combination of operational parameters for the process were identified. However, due to the great number of variables which may have a cumulative effect on biomass output, more sophisticated methods were employed which rely on *a priori* knowledge of the model of the system and its interaction with the environment. Models have recently been developed for the on-line optimisation of *Spirulina* production processes in open ponds (Guterman & Ben-Yaakov, 1989, 1990; Guterman *et al.*, 1990). These models describe, on a macroscale, outdoor *Spirulina* ponds, and can aid in the prediction and optimisation of production processes without the difficulties and tediousness of conventional optimisation procedures.

1.12 CONCLUSIONS

In the last decade, scientific, technological and economic information has proved the viability of continued research into algal biotechnology. *Spirulina*, aside from its application as a protein source, also has marketable application as a dietetic and cosmetic. The two dominant pigments from cyanobacteria, phycocyanin (Spirulina-blue) and phycoerythrin, as well as the

photosynthetic pigment, β-carotene, polysaccharides, arachinodonic acid and ecosapentanoic acid have stimulated economic interest. More than 10 capital ventures or new companies including Cyanotech, Martek Corporation, R&A Plant Soil, Ocean Genetics and Microbial Resources in the USA, Western Biotechnology in Australia, Dainippon in Japan, and Koor Foods in Israel are investing technology and capital in this new market. Industrial agencies such as Battelle in the USA and PA Technology in the UK are also dealing with specific aspects of microalgal exploitation (Gudin, 1988).

CHAPTER 2

RESEARCH PROGRAMME

Spirulina AND TANNERY WASTEWATER

Summary The occurrence of *Spirulina* blooms in tannery effluent evaporation ponds, and the economic advantages of this source over pure-culture grown biomass are noted. Questions into the preliminary investigation of this protein source are highlighted.

The preceding review of the available literature indicates the application potential of *Spirulina*, most noteworthy in terms of human and animal nutrition. The chemical composition of commercially-produced *Spirulina* makes it an ideal protein, vitamin and mineral source. The major handicap impeding *Spirulina* utilisation is the high production costs, which result primarily from the low yield per unit area obtained at present. Capital costs make up 40% of the production costs, in which the cost of pond construction is prominent. In 1980, production costs were estimated at $500 per ton of dry algal mass. This is more than twice the cost of soybean meal, which has a similar protein value (Richmond, *et al.*, 1980). It is estimated (Berend *et al.*, 1980) that pond construction and maintenance, and nutrients supply (media costs) for the production of mass-cultures of *Spirulina* amounts to 36-57% of the total production costs. Becker & Venkataraman (1980) maintain that expenses for cultivation of *Spirulina* in artificial medium are magnitudes too high to become economical. The advantages of easy harvest and processing of *Spirulina* would be annulled by the high cultivation costs.

The observation by Rose (1992) of the occurrence of *Spirulina* blooms in tannery effluent evaporation ponds in Wellington, South Africa, brings to light an as yet unexploited, potentially low production-cost source of this cyanobacterium. Capital costs in the form of pond construction and media preparation would be nullified, and utilisation of this source of *Spirulina* could thus turn an ecologically-disturbing economic dead-end into a commercially

significant enterprise. This study was thus initiated in order to determine the viability of using this source of effluent-grown *Spirulina* in aquaculture.

The economic-return incentive is motivated by the cost of maintenance and disposal of the tannery effluent, a formidable task considering the volume and pollutant load. Furthermore, restrictions on ground seepage raised the initial capital outlay by calling for the installation of pond liners.

Hacking (1986) put forward the proposal to reverse the capital flow by obtaining a product of value from a cost-intensive problem. In this instance it would mean the maintenance of a near mono-algal culture for the collective tasks of pollutant removal, and the economic potential of the algal biomass obtained. The saline nature of the effluent provides a selective pressure for the dominance of halotolerant/halophilic species. The alkaline nature of the tannery effluent provides an even greater selective advantage for the proliferation of the halotolerant, alkalophilic *Spirulina*.

The nutritive properties of pure-culture grown *Spirulina* biomass are well documented. As the evaluation of nutritional and toxicological properties of a novel protein source can be an exhaustive task, the main areas of concern will need to be highlighted in order to determine the preliminary feasibility of the incorporation of this source of *Spirulina* in aquaculture rations.

The following investigations were consequently highlighted:

1. What is the crop yield of this source of effluent-grown *Spirulina*, and why is the ecology of the effluent evaporation ponds so adequately suited to support the growth of this organism?

2. Can this source of *Spirulina* be harvested and processed to a usable form?

3. Is the chemical composition of this source of *Spirulina* comparable to pure-culture-grown biomass?

4. Are there any toxicological constraints on the use of effluent-grown *Spirulina* as a feed source in animal rations?

5. Can this source of *Spirulina* be used in the artificial rearing of the commercially significant South African abalone, *Haliotis midae*?

6. Can effluent-grown *Spirulina* provide an adequate protein supplement in the aquaculture-rearing of the rainbow trout, *Oncorhynchus mykiss,* and does the use of this cyanobacterium result in enhanced colouration of the fish?

CHAPTER 3

OCCURRENCE OF *Spirulina* IN TANNERY EFFLUENT

Summary An assessment of the microbial populations of the tannery effluent evaporation ponds at Western Tanning Co. (Wellington, S.A) was undertaken. The dominant photosynthetic organism was the commercially significant cyanobacterium, *Spirulina*. Analysis of the effluent provided a correlation between the prevailing chemistry of the ponds and the occurrence of *Spirulina*.

3.1 INTRODUCTION

Tanneries have been described as producing the most polluting wastes of any industry (Tsotsos, 1986). Leather tanning is carried out batchwise and involves many stages of processing. The traditional and most widely used method of processing hides is by salt curing, which produces about 8 L of near-saturated brine per hide (Cooper *et al.*, 1984). It is estimated that South African tanneries use approximately 600 000m^3 water, almost all of which becomes effluent (Rose, 1992).

Hide tanning results in a final effluent whose rate of discharge and chemical composition varies. The upstream effluents derived from the various chemical processes also differ widely in terms of their pollutant characteristics. The majority of the pollution load in the ponded downstream effluent arise from the beamhouse and tanning processes (Jackson-Moss, 1990).

The beamhouse processes involve:

hide soaking	dirt, salt, blood, manure, urine, grease, proteins and emulsifiers
unhairing and liming	hair, proteins, Na_2S, lime
dehairing	hair
fleshing	fleshings, proteins, fats
splitting	dust, hide shreds
deliming and bating	ammonium and calcium salts, enzymes, inorganic and organic acids

The tanning processes involve :

pickling	salt, inorganic acids
tanning	Cr^{3+} salts, vegetable and synthetic tannins, organic acids (Bailey, 1977)

Tanneries normally combine all the individual waste streams to produce a total mixed effluent, characterised by high concentrations of organics, suspended and dissolved organic or inorganic solids (of which salt is a noteworthy contributor), and a variety of other adulterant contaminants.

As with all tanneries, WTC uses water in all forms of the leather processing industry. The processes are generally non-consumptive, apart from minor, irrecoverable losses by evaporation, steam, etc. It is estimated that between 86 and 95% of the water used is recovered as effluent (Jackson-Moss, 1990). As leather manufacture is carried out batchwise, and involves many different stages of processing, the resulting effluent may vary considerably throughout the day. The ponded effluent, representing the total mixed effluent, is characterised by high concentrations of organic matter (proteins and fats), suspended solids and dissolved solids.

Environmental pressures necessitated the development of pre-treatment processes before discharge to ponding systems (Tsotsos,1986). The establishment of activated sludge and oxidation ditch processes have contributed considerably to the generation of dischargeable or recyclable effluents (Bailey, 1977).

Where discharge to the municipal sewer is not permissible, evaporation ponding systems have been used as an additional component of facultative lagooning (Rowswell *et al.*, 1984). Evaporation ponding of the effluent allows for the evaporative loss of water in a series of large-area ponds, resulting in a concentrated brine solution in the final ponds. The salinity gradient existing across the treatment series allows for the growth of mixed cultures of algae and bacteria.

Such an evaporation ponding system is currently in operation at WTC (Wellington). These ponds periodically erupt in population blooms, with the dominant photosynthetic populations being *Spirulina* (Cyanophyceae) and *Dunaliella* (Chlorophyceae). This has recently been reported by Rose (1992) in a preliminary study of the evaporation ponding effluent treatment system in operation at the Western Tanning Co. (WTC), Wellington, South Africa.

It has been noted that species dominance in the wastewater depends primarily on the organic loading (Palmer, 1969). Highly polluted anaerobic water permits the development of *Euglena* and *Chlamydomonas* species only. Then, in order of decreasing organic load, appear *Scenedesmus*, *Chlorella* and *Micractinium* spp. Development of blooms of other green algae, phytoflagellates, cyanobacteria and diatoms are generally indicative of low organic load (Abeliovich, 1986).

3.2 RESEARCH OBJECTIVES

Given the identification of production costs as one of the main limitations in *Spirulina* production, the proliferation of *Spirulina* in the evaporation ponds at WTC could thus present an economically feasible production system, as pond and media costs fall away. The only other costs involved are those of harvesting, dewatering and drying, and, indirectly, the costs

of research and labour. A preliminary study of the WTC was then embarked upon, with the following questions in mind:

1. Which microbial populations are present in this niche?

2. What is the standing crop and yield potential of the *Spirulina* biomass in the evaporation ponds?

3. Why does this source of effluent provide such an adequate medium for growth of *Spirulina?*

3.3 MATERIALS AND METHODS

3.3.1 Study Site

The evaporation-ponding effluent-treatment system at Western Tanning Co. (WTC), Wellington, South Africa, was used in this study.

3.3.2 On-site Sampling and Assessment

A microbiological evaluation with tentative identification to the genus level was performed, using morphological descriptions from Bergey's Manual (Staley *et al.*, 1989).

The standing crop of *Spirulina* biomass was determined by filtering, drying (60°C, 12 hrs) and weighing known volumes of effluent.

An evaluation of the ponded effluent was undertaken using methods outlined in APHA Standard Methods (1980) and Jackson-Moss (1990). Analytical procedures included the following:

pH
Conductivity
Permanganate Value (PV)
Chemical Oxygen Demand (COD)
Total Dissolved Solids (TDS)
Total Dissolved Inorganic Solids (TDIS)
Suspended Solids (SS)
Total Kjeldahl Nitrogen (TKN)
Free & Saline Ammonia as NH_3
Sulphide as S
Nitrates
Chloride as Cl
Sulphate as SO_4
Sodium as Na
Magnesium as Mg
Calcium as Ca
Chromium as Cr
Boron as Bo
Potassium as K
Phosphorus as P_2O_5
Iron as Fe
Aluminium as Al
Molybdenum as Mo
Cobalt as Co
Cadmium as Cd

Mercury and Selenium could not be determined due to problems experienced with the atomic absorption standard spectra of these metals.

3.4 RESULTS

3.4.1 Study Site

The treatment process currently in use at WTC employs a cascade of 15 ponds (Figure 3.1), a system which has been in operation since the mid-1960's (Rose, 1992). The ponds are designed to treat 270 m^3/day, and constitute a surface area of 13.6 ha, a capacity of 197 000 m^3, and vary in depth from 0.5-3 m. The system dimensions for the various ponds are shown in Table 3.1 (after Rose, 1992).

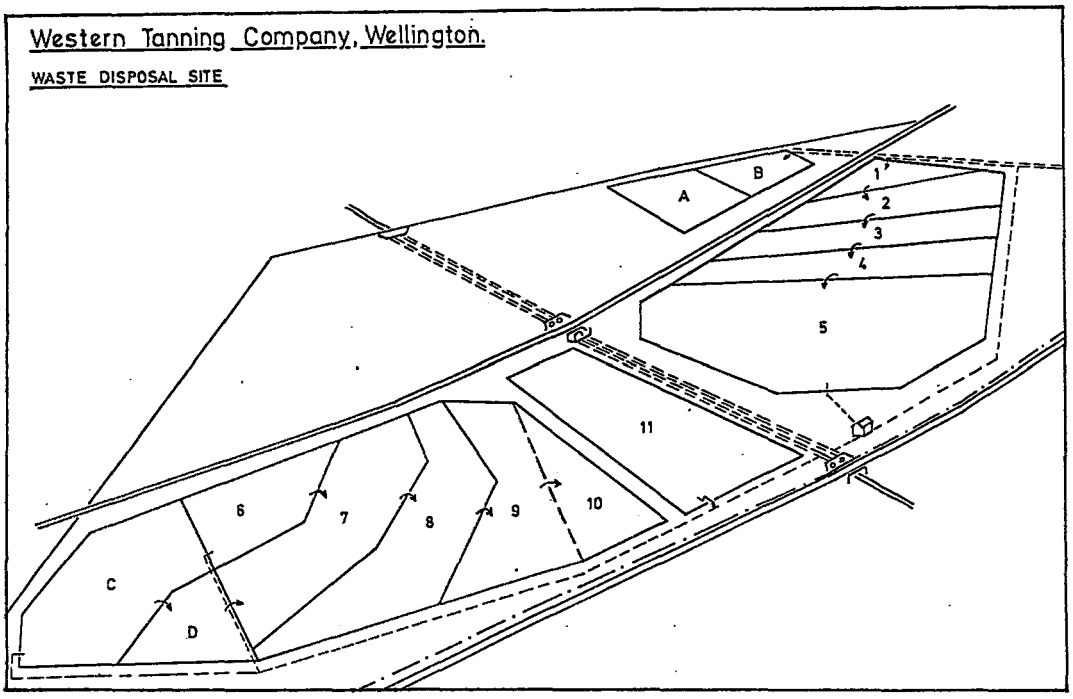

Figure 3.1 Plan view of the evaporation ponding cascade at WTC, Wellington. Effluent is pumped from the factory to pond 1, flows to pond 5, where it is pumped to pond 6, and then concentrated by evaporation through to ponds 10 & 11. At certain times, ponds A and B remain stagnant, while the other ponds are used in the evaporation cascade.

Table 3.1 System dimensions for the evaporation effluent-treatment ponding system in operation at WTC, Wellington.

Pond	Area (in m^2)	Capacity (in m^3)
A	9 477	19 980
B	5 354	8 020
1	4 833	69 088
2	9 667	
3	8 923	
4	9 295	
5	26 097	
6	3 253	12 000
7	7 250	
8	9 669	
9	5 454	
10	2 370	
11	16 359	49 077
C	13 100	25 900
D	5 700	13 100
Total	136 801	197 165

Subsequent to the design of this system, licensing by the Department of Water Affairs (DWA) for the operation of a closed site requires the collection of all seepage and rainfall run-off from the entire 40 ha site to be pumped into the ponding system. The existing ponding system presently encounters problems in coping with the additional flow.

3.4.2 On-Site Sampling and Assessment

The predominant bacteria present in the initial ponds were the purple archaebacteria, *Halobacterium spp.*, giving certain of the ponds a characteristic purple-red colour (Figure 3.2).

Figure 3.2 Red colour observed in the initial ponds in the effluent-treatment cascade. The colour is caused by the predominant halobacteria.

Both photosynthetic and non-photosynthetic bacteria were also found to occur in the effluent, especially abundant in the sediments. Tentative identification show that the predominant bacteria are the purple *Chromatium sp.*, and the green *Chlorobium sp.*. These organisms reveal their presence macroscopically by flocculating on the surface as a white sheen-film of sulphur.

The predominant photosynthetic species in the latter ponds are the cyanophyte *Spirulina* and the chlorophyte *Dunaliella*. *Spirulina* autoflocculates to the surface and is wind-concentrated at the pond edges, giving rise to the characteristic 'bloom' (Figure 3.3). The *Spirulina* population consists of more than one species, or a number of morphological variants of the same species. The species may be either *S.platensis* or *S.maxima*, or morphological variants of either of these two species (Figure 3.4).

Figure 3.3 Rafts of *Spirulina* in Pond D in the evaporation ponding cascade.

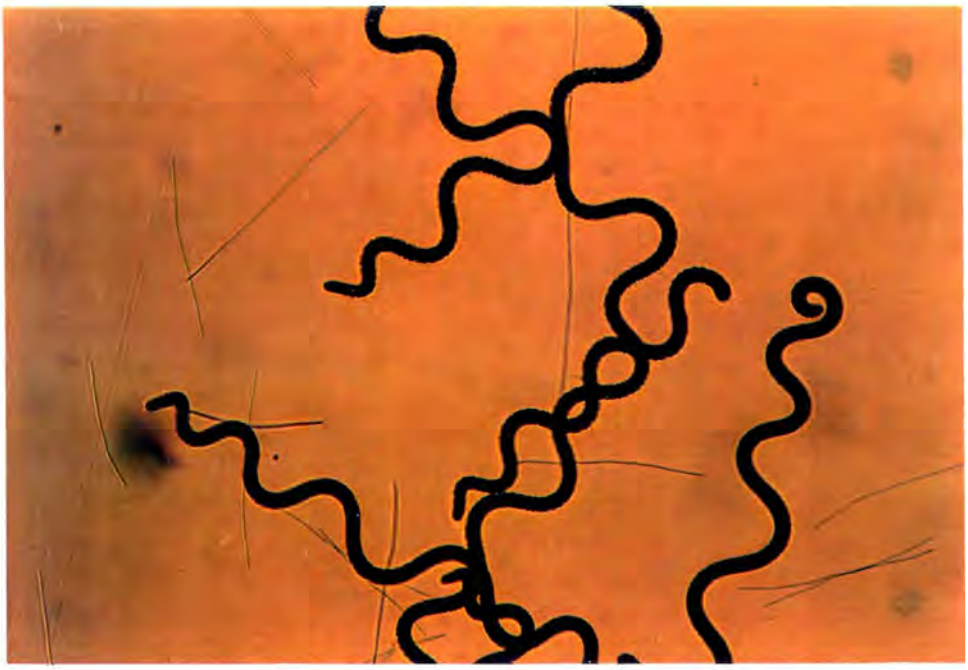

Figure 3.4 *Spirulina sp.* in ponded effluent. Note the presence of the coexisting filamentous cyanobacterium, *Anabaena* spp.

A dynamic flux seems to exist between the various populations inhabiting this niche. *Spirulina* moves through the entire vertical water column for at least some part of the day. Flocculated mats are at their most dense during midday, sinking during early morning and evening, revealing the red colour of the co-existing halobacteria. On average, however, there seems to be an even distribution of *Spirulina* in the vertical water column.

A mixed pond D sample was found to contain a 0.2% solids concentration. The surface mat had a 2.2% solids concentration. Autoflocculation of *Spirulina* therefore results in a ten-fold increase in solids.

Ponds D, 7, 8 and 11 supported the densest population of *Spirulina*. A typical evaluation of the standing crop of *Spirulina* biomass in these ponds is as follows (Table 3.2):

Table 3.2 Standing crop of *Spirulina* in evaporation ponds during March, 1992. Values are averages of a number of sampling sites, and at depths 0 and 0.3 m.

Pond	Biomass Conc. ($g.L^{-1}$)	Standing Crop (kg)
D	4.69	6 143.9
7	8.72	2 720.6
8	5.97	2 474.0
11	5.20	25 520.0
TOTAL	24.58	36 858.5

There is thus a standing crop of nearly 37 tonnes in 38 978 m^2. This corresponds to a biomass concentration of 9.5 t/ha (dry weight).

In order to discover possible relationships between species dominance and prevailing chemical characters in the different ponds, an evaluation of the effluent chemistry was performed. Table 3.3 shows the possible variations between ponded effluent of the 15 ponds at different times. The samples were drawn and analyzed at two month intervals (August & October, 1990).

Table 3.3 Analysis of ponded effluent during Aug. & Oct., 1990.

Pond	Colour		Odour		pH		Cond.x100 mS/cm^{-1}		TDS %	
	Aug	Oct	Aug	Oct	Aug	Oct	Aug	Oct	Aug	Oct
A	BP	Gr	-	++	8.9	7.8	40	28	3.2	2.0
B	BP	B	-	-	8.9	9.3	38	41	2.9	3.3
1	Gr		++		8.2		31		2.3	
2	Gr	BP	++	++	8.0	8.1	30	29	2.2	2.1
3	Gr	P	++	++	8.0	8.2	29	32	2.1	2.5
4	Gr	P	+	+	8.0	8.5	26	36	1.9	2.8
5	Gr	BP	-	+	8.1	8.8	23	27	1.6	2.0
6	G	G	0	0	9.5	9.8	50	53	4.3	4.5
7	G	G	0	0	9.0	9.9	45	50	3.6	4.2
8	G	G	0	0	9.2	9.9	46	54	3.8	4.6
9	G	G	0	0	9.2	10	45	59	3.7	5.2
10	G	G	0	0	9.2	10	45	44	3.7	5.2
11	G	G	0	0	9.5	9.9	42	44	3.4	3.6
C	BP	G	0	0	9.4	9.7	43	44	3.5	3.5
D	BP	G	0	0	9.2	9.8	46	48	3.8	4.0

Colour Gr=Grey G=Green B=Brown BP=Brown Pink P=Pink
Odour ++=Bad +=Noticeable -=Not Unpleasant 0=None

Pond	Sol. PV mg.L^{-1}		Sol.COD g.L^{-1}		SS mg.L^{-1}		Sol. N mg.L^{-1}		Cl %	
	Aug	Oct	Aug	Oct	Aug	Oct	Aug	Oct	Aug	Oct
A	341	368	2.8	4.5	82	94	105	703	1.4	0.7
B	300	135	2.4	1.0	108	46	129	87	1.3	1.5
1	402		4.1		240		426		0.9	
2	371	560	4.2	3.6	246	136	462	606	0.9	0.9
3	310	559	2.5	4.2	248	154	414	414	0.9	1.0
4	213	442	1.8	3.5	200	173	392	258	0.7	1.2
5	269	245	2.3	1.9	144	86	165	143	0.7	0.9
6	284	69	2.7	0.5	70	46	31	39	1.9	2.1
7	83	112	1.4	1.0	26	44	24	29	0.8	1.9
8	173	51	1.7	0.4	112	108	28	27	1.7	2.2
9	82	44	1.6	0.4	4	34	21	27	1.6	2.5
10	142	44	1.7	0.4	4	46	20	27	1.6	2.5
11	147	49	2.0	0.5	8	40	20	27	1.5	1.7
C	112	49	1.0	0.4	12	56	29	29	1.5	1.4
D	97	49	1.2	0.4	10	62	31	22	1.6	1.7

Pond	Na_2S $mg.L^{-1}$		SO^4 $mg.L^{-1}$		TDIS %		K $mg.L^{-1}$	
	Aug	Oct	Aug	Oct	Aug	Oct	Aug	Oct
A	29	936	841	2339			221	86
B	16	54	957	722			208	134
1	472		1795				163	
2	113	569	1990	2279			152	94
3	222	603	1485	275			148	108
4	94	381	1623	1359			141	122
5	27	225	783	644			136	91
6	<1	<.5	831	1337			265	182
7	<1	<.5	883	1010			239	166
8	<1	0.8	911	975	3.18	2.42	248	194
9	<1	<.5	844	1080	3.21	3.21	244	218
10	<1	<.5	871	1319	3.14	3.19	241	212
11	<1	<.5	934	920	2.76	2.22	224	150
C	<1	<.5	711	934	2.86	2.15	235	148
D	<1	<.5	908	959	3.21	2.47	247	181

As can be seen, variation does exist between the ponds, and between the same ponds at different times. The most noticeable trend, however, is a reduction in organic content in the latter ponds (indicated by lower PV, COD, SS and N values). This reduction in organic load is accompanied by a reduction in sulphur, an in increase in pH, and a concomitant increase in the cyanobacterial biomass (denoted by the green colour). There is thus a direct relationship between the prevailing chemical conditions and the dominant microorganism present.

3.5 DISCUSSION

The downstream effluent in any industrial system depends on the product produced. In the tanning industry, various processes are combined to produce an effluent characterised by high levels of salinity, organic load, sulphide and alkalinity (Rowswell *et al.*, 1984)

It was suggested by Rowswell & Rose (1990) that institution of an active algal treatment process and harvesting the algae present in the ponding system offers the advantage of removing N,P and K from the final effluent. This would reduce the risk of eutrophication in downstream impoundments if the release of saline concentrates with stormwater flows was to be licensed.

The initial ponds are characterised by halobacterial dominance, indicated by the red colour. These organisms can function in the photoautotrophic mode (under aerobic, illuminated surface conditions) and the heterotrophic mode (utilising amino and organic acids, sodium and sulphide, in the water column and in the sediments of the ponds) (Brock & Madigan, 1988). The initial ponds thus satisfy the stringent conditions required by this group of bacteria. The organic load is at its greatest in the initial ponds (facilitating halobacteria and sulphur-bacteria heterotrophic growth), as are the other elements essential for sustenance of the halobacteria.

The latter ponds are characterised by *Spirulina* and *Dunaliella* growth. More than one form of *Spirulina* appears to be present, characterised by varying degrees of helical coiling. These may either be different species (probably *S.maxima* and *S.platensis*), or they may be morphological variants of the same species. Three distinct morphological variants of *Spirulina* have been noted (Richmond, 1988). These different morphology types have also been documented by van Eykelenberg (1979, 1980), in response to the prevailing physical conditions, specifically light intensity and temperature. It is thus possible that the different forms noticed in the effluent are caused by the vertical stratification of light penetration and temperature, although the distribution of *Spirulina* through the entire water column does not support this theory.

Through the cascade of evaporation ponds, there is a shift in both chemical composition of the effluent and the dominant microorganism population. Initial ponds, characterised by low pH and TDS, and high COD, SS, soluble N, Na_2S and SO_4, are colonised by halobacteria. The latter ponds exhibit chemical characteristics at the other extreme, and, as are characterised by algal/cyanobacterial dominance. *Spirulina*, a mesohaline species, dominates at salt concentrations $< 30 g.L^{-1}$. *Dunaliella*, a halophilic chlorophyte, predominates at higher salt concentrations.

The latter ponds are characterised by high alkalinity, a known selection factor for the proliferation of *Spirulina*.

Organic uptake, initially thought to be due entirely to bacterial oxidation, may also occur via *Spirulina*. The phenomenon of obligate heterotrophy has been demonstrated in a variety of cyanobacteria (e.g. Smith *et al.*, 1967; Diakoff & Scheibe, 1975; Hoare *et al.*, 1967; Rippka, 1972; van Baalen *et al.*, 1971). The possibility therefore exists that organics in the effluent enhance *Spirulina* growth due to organic uptake by the cyanobacterium. This hypothesis is also postulated by Soong (1980) who found that addition of digested hog manure effluent to the *Spirulina* medium can greatly enhance biomass yields.

Thus, although it seems convenient to assume localization of nutritional modes, this probably does not occur. Overlapping of both auto- and heterotrophy occurs in a single population. A great deal of information is available on the symbiotic relationship between algae and bacteria in oxidation ponds (Ganapati, 1975; Oswald, 1988a). Generally, the principle products of bacterial organic oxidation are CO_2, NH_3 and H_2O, which constitute the main requirements for algal photosynthesis (with the additional requirement of light energy).

It is documented (Palmer, 1969) that algal species dominance depends primarily on the organic load. Development of blooms of cyanobacteria is generally indicative of a low organic load. This is shown in the evaporation cascade, where *Spirulina* blooms dominate in ponds with the lowest organics concentrations.

The blooms of *Spirulina* in the tannery effluent evaporation ponding system represents a succession of the water from oligotrophic (low nutrients) to euphotic (nutrient rich) conditions. Euphotic waters are accompanied by an increase in algal productivity, and the development of seasonal or persistent blooms. The competitive edge enjoyed by *Spirulina* may be due, in part, to the lower N:P ratio in the water caused by enrichment of the water with phosphorus. The resulting bloom of *Spirulina* floats to the surface, where high irradiance and oxygen concentration leads to their photodynamic death and decomposition. This, in turn, may lead to bacterial utilisation of virtually all of the dissolved oxygen, and to

the development of subsurface anoxic conditions, accompanied by sulphide deposits and the characteristic H_2S odour.

Cyanobacteria employ a variety of strategies to displace other groups of algae (Walsby & Klemer, 1974; Ganf & Oliver, 1982; Paerl & Ustach, 1982; Paerl *et al.*, 1983). The most important of these is the ability to fix nitrogen, thereby overcoming low nitrogen concentrations. Certain cyanobacteria, as is the case with *Spirulina*, optimise nutrient and light gradients by alternate sinking and floating, accomplished by the formation and collapse of gas vesicles (Walsby & Klemer, 1974).

Paerl & Ustach (1982) postulate that the surface scum formed by cyanobacteria results in optimal conditions for photosynthesis and nitrogen fixation, while at the same time successively competing with other algae left in virtual darkness below the surface bloom.

Cyanobacteria have also been shown to excrete substances detrimental to competing species (Dubinsky, 1986).

It is also suggested that cyanobacteria are efficient at fixing CO_2 from low concentrations (Shapiro, 1973). Thus, under conditions of high pH (as in enriched lakes and the ponded effluent under study), cyanobacteria are able to dominate other algae. This hypothesis was tested and confirmed by Shapiro (1973), and seems to apply directly to the phenomena of *Spirulina* dominance over green algae exhibited by the latter ponds in the evaporation cascade. High pH's are also indicative of high oxygen concentrations (i.e. high rates of photosynthesis). A combination of these conditions may result in photooxidative damage (Abeliovich, 1986). This potential harmful effect can be overcome by mixing of the effluent liquid.

The hypothesis of the sequence of events leading up to *Spirulina* dominance can thus be summarised as follows: Productivity increases as nutrients are pumped in with the influent liquid. When the available supply of free CO_2 is used up, soluble bicarbonate is utilised. The pH rises, and the more efficient CO_2 utilization capacity of *Spirulina* allows this species to dominate (ponds C-11).

Ammonia is probably used as the nitrogen source by *Spirulina*. Although it is documented that nitrates are the main nitrogen source assimilated by *Spirulina* (Richmond, 1988), the low nitrate levels (< 1mg/l) and the high ammonia level (111mg/l) present in the effluent, points to the probability that ammonia is the utilised nitrogen source. Oxidation of ammonia to nitrate rarely occurs, because ammonia is assimilated by the organism, lost to the air, or precipitated due to the high pH before nitrification is established. This was also observed by Ganapati (1975) in a number of algal cultures. However, much of the ammonia either remains in the effluent, or is utilised by the algae. Major loss is prevented by the high NaCl concentration. This phenomenon was also observed by Soong (1980). *Spirulina* has also been shown by various authors to utilise a variety of other nitrogen sources. The mixed effluent presents a variety of possible nitrogen sources, which may be partly responsible for enhanced growth.

The latter ponds, dominated by *Spirulina* growth, are also characterised by lower sulphur concentrations, in terms of both Na_2S and SO_4 content. It is possible that the sulphur is being utilised by *Spirulina*, as the presence of two active permeases have been noted in *S.platensis* (Menon & Varma, 1982). A gene regulating sulphur uptake in the cyanobacterium *Synechococcus* has also been isolated and identified (Laudenbach *et al.*, 1991). These genes have been implicated in active uptake of sulphur (Laudenbach & Grossman, 1991).

The phosphate uptake kinetics also favour cyanobacterial growth, as noted by Shapiro (1973).

Bio-stimulatory nutrients are available in abundance in the effluent. Essential macronutrients include C, H, O, N, K, Ca, Mg, S and P. Essential micronutrients present include Fe, Mn, Cu, Zn, Bo, Na, Mo, Cl, and Co.

The prevailing high daytime air temperatures in Wellington (social proof provided by the colloquially used 'Hellington') ensure near-maximal growth of *Spirulina*. The optimal temperature for growth has been documented between 35 & 37°C. The minimum permissable temperature that allows some growth is 18°C (Richmond, 1988). It is also known that *Spirulina* can tolerate relatively low night temperatures (Richmond *et al.*, 1980).

Light intensity has been documented as one of the primary factors influencing the biomass output rate. However, manipulation of the conditions affecting light penetration necessitates the installation of a mixing device.

At present, there is a standing crop of 37 tonnes of *Spirulina*. Pure-culture *Spirulina* is currently being marketed at up to $19/kg by Earthrise Farms in California. Working on this estimate, the effluent ponds at Wellington have a standing crop worth nearly $^3/_4$ million US dollars.

CHAPTER 4

HARVESTING AND PROCESSING

Summary An investigation into the use of a screen harvester for the concentration of *Spirulina* was performed. The first small-scale harvester with a 100 μm mesh was used successfully in a ten-fold concentration of *Spirulina*, yielding a solids concentration of 21.4% (dry weight). The biomass was sun-dried, obtaining a yield of 25 kg. The subsequent design and evaluation of a scale-up, technical-scale model with an 80 μm mesh yielded similar cell concentrations, with a biomass yield of 250 kg.

4.1 INTRODUCTION

The biotechnological utilization of micro-algae depends, to a large extent, on the economics and feasibility of the harvesting technique (Abeliovich, 1986; Mohn, 1988).

Costs associated with harvesting include the acquisition, installation and maintenance of the equipment, power consumption by the device, and high costs of thermal energy requirements for final product processing and drying (Mohn, 1980).

4.1.1 Microalgal Harvesting

Several widely differing technologies have been developed for the separation of a variety of micro-algae from their growth medium, and have been extensively reviewed (Ben-Amotz & Avron, 1989; Golueke & Oswald, 1965; Mohn, 1980; 1988). Although mention is made of pure-culture-grown biomass, most of the attention has focused on the removal of waste-grown biomass from HROP systems treating municipal effluents in order to comply with environmental discharge standards (Rose, 1992).

Cell separation methods currently in use include centrifugation (Mohn, 1980), electroflocculation (Richmond & Becker, 1986), chemical flocculation (McGarry, 1970; Golueke & Oswald, 1965), sedimentation (Mohn, 1980), air flotation (Richmond, 1986d),

continuous belt filtration, vibrating and stationary screens, sand bed filtration (Richmond & Becker, 1986), and a variety of membrane-separation technologies (Rose, 1992). Only a few of these systems, however, have potential as efficient, low-cost harvesting methods (Mohn, 1980), and a few of the more important ones are discussed below.

4.1.1.1 Centrifugation

Centrifugation is the most direct method for removing algal cells from the growth medium (Mohn, 1988), and a near-100% efficiency is usually possible. Mohn (1980) evaluated a range of centrifuges including a plate separator, nozzle centrifuge, screw centrifuge and hydrocyclone. Cultures can usually be concentrated to 15-20% solids (dry weight).

Centrifugation can be used equally efficiently with both filamentous and non-filamentous microalgae. *Spirulina*, which is rich in air vesicles, tends to rise in the centrifuge rather than settle, and can be removed as a floating cream (Richmond & Becker, 1986).

The major advantage of centrifugation is its simplicity. This, however, is overridden by the high investment cost and energy demand, which makes centrifugation impractical for the mass production of inexpensive algal biomass (Richmond & Becker, 1986). Corrosion of the equipment is also a problem when saline media is used.

4.1.1.2 Sedimentation

Tank sedimentation has the disadvantage of requiring special structures, necessitating considerable additional space and financial input requirements. Moreover, sedimentation is usually only effective with the addition of flocculating agents. Non-toxic flocculating agents such as potato starch derivatives and chitosans are suitable for initiating sedimentation (Mohn, 1988).

4.1.1.3 Flotation

Flotation processes have been found to operate more efficiently and rapidly than does sedimentation, and also achieve a higher solids fraction (up to 7%). Disadvantages of flotation include the high costs and additional energy requirements for pumps and generation of compressed air. If the costs for flocculation agents are included, then the costs of harvesting by flotation is similar to that of centrifugation (Mohn, 1988).

Certain blue-green algae float and sink intermittently due to gas vacuoles (Walsby, 1977). This facilitates simple harvesting by skimming (Koopman & Oswald, 1977, cited in Oswald, 1988b).

4.1.1.4 Filtration

The economics of filtration as a separation technique compares favourably with most other techniques (Hacking, 1986). A variety of filtration technologies with potential in micro-algal harvesting have been evaluated. These include, amongst others, sand filtration (Oswald, 1988b; Naghavi & Malone, 1986) and cloth filters (Mohn, 1988). A range of filter presses, belt presses, pressure suction filters, vacuum drum filters and diaphragm presses have also been used in conjunction with a variety of precoats (Mohn, 1988).

A wide range of filters have been evaluated, but they all suffer from the universal problem of rapid clogging and fouling (Oswald, 1988b). Continual backwash of the filter is thus needed.

With the advent of micro- and ultra-filtration, and the improvement of membrane technology, a new sphere to this part of the biotechnological exploitation of microalgae has been added. Cross-flow filtration has been used in a variety of separation and concentration processes. The first report of the feasible use of these devices in micro-algal harvesting has recently been made, with the problem of clogging being alleviated by the continual shearing force of the influent liquid (Maart *et al.*, 1990).

4.1.1.5 Flocculation

Harvesting of microalgae is simplified when the algal cells form aggregates/visible flocs that sediment or float. Flocculation may be spontaneous (as may be caused by a sharp change in the pH or cation concentration), or an inherent physiological phenomena.
Algae capable of autoflocculation have an inherent advantage in the field of algal biotechnology, as harvesting costs are significantly reduced (Borowitzka, 1988). Mass culture experiments on algae exhibiting this phenomena have been performed. Species evaluated include the filamentous red marine algae *Acrochaetium sp.* and *Asterocystis sp.* (Groenweg, 1978, cited in Borowitzka, 1988), and freshwater algae such as *Hormidium*, *Hydrodictyon*, *Oscillatoria*, *Stigeoclonium*, *Ulothrix* and *Uronema* (Tamiyama, 1957).

The filamentous nature of *Spirulina* is one of the main reasons for the success of this organism in mass culture (Borowitzka, 1988). These organisms have a granular cytoplasm containing gas vacuoles (Richmond, 1986b), and are capable of autoflocculation, physical proof shown by the mats of *Spirulina* forming scum layers in ponds under certain conditions (Ciferri, 1983). The air vesicles, large size and spatial orientation of *Spirulina* thus gives it the ideal morphology and physiology for autoflocculation.

Flocculation may also be physically or chemically induced. Physicochemical separation processes are affected mainly by algal shape and size. *Micractinium*, an algae which forms microcolonies and possess setae, flocculate more readily with chemical flocculants (such as alum) than does *Chlorella*, a small, unicellular algae (Azov *et al.*,1980).

4.1.1.6 Screening and Straining

Many larger filamentous organisms, like *Spirulina*, can easily be separated by inexpensive screens or sieves (Mohn, 1980; Mitchell, 1986). Screen types that are applicable to microalgae include vibrating screens, rotating screens, microstrainers, and cascade screens (Richmond & Becker, 1986). The advantage of screen filters is that a pure product is obtained, as all other micro-organisms are usually too small to be retained by the relatively large mesh sizes required for the separation of filamentous forms from their growth medium.

These screens have been successfully used in the concentration of the cyanobacteria *Oscillatoria* and *Spirulina* (Richmond & Becker, 1986; Mitchell, 1986), and the chlorophyte *Uronema* (Sinchumpasak, 1980)

Corrosion problems have been overcome by the use of plastic materials such as nylon and teflon. Clogging by slime, however, remains the greatest problem.

Harvesting of *Spirulina* can be accomplished by vibrating screens. If the filaments are long enough, up to 95% harvesting efficiency is possible. The disadvantage of vibrating screens is the rubbing effect on the filaments, leading to cell disruption and an increase in the organic load of the pond (Richmond & Becker, 1986).

Generally though, the method that produces pure algae reliably and with least cost is microstraining. This method works particularly well with filamentous types such as *Spirulina*, but also works well with the larger species such as *Scenedesmus*, or small organisms with extensive setae, such as *Micractinium* (Oswald, 1988b).

Another problem associated with the use of screening devices, is that they preferentially remove the larger algal species, thus enriching the medium with smaller contaminating species. *Chlorella*, a unicellular cyanophyte, is a common contaminant of *Spirulina* cultures, and removal of the *Spirulina* filaments by screening may lead to a species shift to *Chlorella* in the enriched medium (Richmond & Becker, 1986).

If the harvesting process is accompanied by even a small amount of cell breakage, the returning flow is sufficiently enriched in organic matter which may result in the succession by mixotrophic or heterotrophic competitors (Richmond, 1988). As noted, then, the best mode of harvesting the algae should meet two criteria: all the biomass should be removed from the medium, and no cell damage/breakage should occur. Such a harvesting system is not yet available to the *Spirulina* industry (Richmond, 1988).

4.1.2 Biomass Drying

In the commercial production of *Spirulina*, drying costs may constitute up to 30% of the production costs. Different drying systems differ in terms of capital investment and in energy requirements, and have a marked effect on the food value and taste of the product (Richmond, 1988).

The harvested slurry should preferably be rinsed in acid water (pH 4.0) to remove adsorbed carbonates, and thus reduce the ash content (Richmond, 1988).

The usual method for the drying of *Spirulina* is spray-drying. Drum-drying also yields an acceptable product in the form of flakes. Venkataraman *et al.* (1980) evaluated a number of drying methods, including spray-fed electrically heated single drum drying, steam heated double drum drying, vacuum shelf drying, and sun-drying. They found that the residual moisture content was the lowest with the steam-drum method when compared to the electric-drum method, and also has the lower production cost of the two. Although the vacuum shelf dried product also has a low residual moisture content, its porous structure makes it more hygroscopic.

Although direct sun-drying is feasible, the final product is not desirable for human consumption, because of the unpleasant odour, and the slowness of the dehydration process which allows degenerative processes to set in. Sun-drying also takes place at relatively low temperatures when compared to processes such as drum drying (120°C for a few seconds). The sun-dried product may consequently exhibit a higher bacterial count (Richmond, 1988).

Sun-dried *Spirulina* can, however, be used successfully in the preparation of animal feeds (Richmond, 1988). Since *Spirulina* has no cellulose cell wall. it can be sun-dried without any problem of interfered digestibility. Because of the length of time needed to dry the slurry, a solar heater has been devised by Venkataraman *et al.*, (1980). The heater consists of a Deal wood box coated black with upper glass plates. Two aluminium plates on either side act as reflectors. They found that a drying temperature of 70°C could be obtained when the

atmospheric temperature was 35°C. *Spirulina* slurry could then be dried in 4-6 hrs, instead of the usual days previously needed.

4.2 RESEARCH OBJECTIVES

The following research objectives were identified:

1. To design, construct and optimise a screen harvesting device for the concentration of *Spirulina* biomass from the evaporation ponded effluent, and to use this device in a small-scale harvest of effluent-grown *Spirulina*.

2. To design and construct a scale-up model of the small-scale screen harvester, and to use this device in a technical-scale harvest.

3. To design a suitable sun-drying apparatus for final dewatering of the harvested *Spirulina* biomass.

4. To process the harvested, dried biomass to a usable form.

4.3 MATERIALS AND METHODS

4.3.1 Small-scale Harvest

An initial small-scale harvest was performed on-site at WTC, Wellington. A screen harvester was constructed according to Mitchell (1986), and is shown diagrammatically in Figure 4.1. A metal frame was clamped into position, allowing for a mesh of 1 m^2 to be stretched horizontally taut across the structure. A nylon mesh of 100 μm mesh size proved the most efficient, in terms of maximal harvest yield with minimal clogging. The screen was diagonally taut, to ensure that the effluent would flow straight down the screen, and not at an angle across it, which would result in effluent spillage over the lateral edges of the screen. The lower, front support rod was set in running grooves in the side supports, to allow for manipulation of the angle of the collecting reservoir.

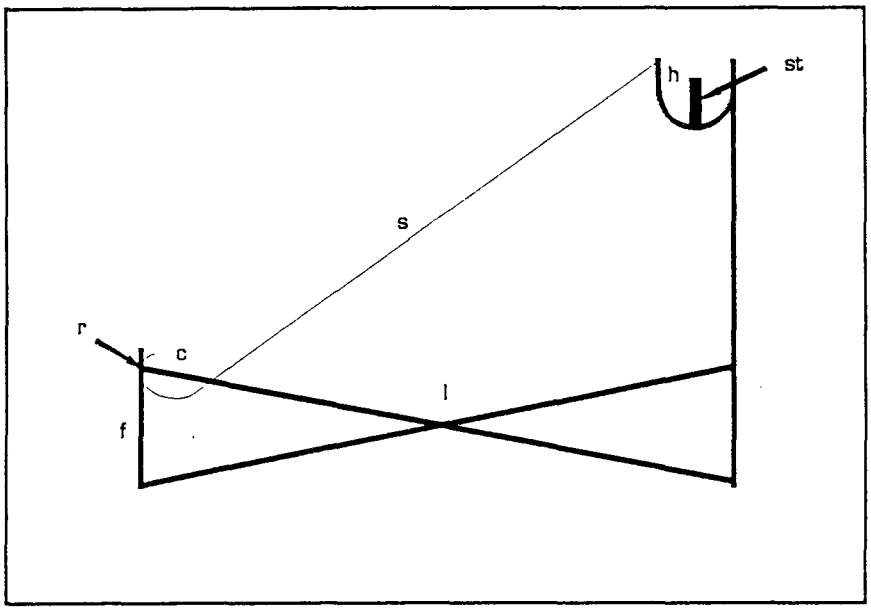

Figure 4.1 Diagrammatic representation of the small-scale screen harvester used for the concentration of *Spirulina* (holding reservoir, collecting reservoir, stilling baffle, screen, lateral supports, front supports, running groove).

The upper holding reservoir contained a base-secured stilling baffle to ensure laminar flow of the effluent onto the screen, and to prevent splashing onto the screen. The upper holding reservoir was over-filled with the *Spirulina* autoflocculated cell slurry (2.2% solids concentration) skimmed from the surface of pond D. Pond D was chosen because of the prevailing *Spirulina* bloom, and because of the level area immediately adjacent to it, which facilitated erection of the harvester. The effluent was then allowed to overflow onto the screen in a virtual mono-layer. The concentrated slurry of the filtered effluent was collected in the angled collecting reservoir at the base. Altering of the collection angle resulted in varying harvest-time efficiencies, but the optimum angle was eventually found to be approximately 45° to the vertical plane. Polyethylene sheeting was placed below the harvester to allo··· the effluent permeate to run into the adjacent pond 7. The starting biomass slurry was skimmed from the surface floc. The on-site operation of this device is shown in Figure 4.2.

Figure 4.2 On-site operation of the screen harvester in a small-scale harvest of *Spirulina* biomass from tannery effluent.

Constant monitoring of the harvesting process was required, as the concentrated slurry was collected manually.

Biomass determinations were performed on a dry weight basis.

4.3.2 Technical-Scale Harvest

A scale-up of the initial small-scale harvester was designed by Roger Rowswell (LIRI Technologies, Grahamstown, South Africa). The side, plan and front elevations are shown in Figure 4.3.

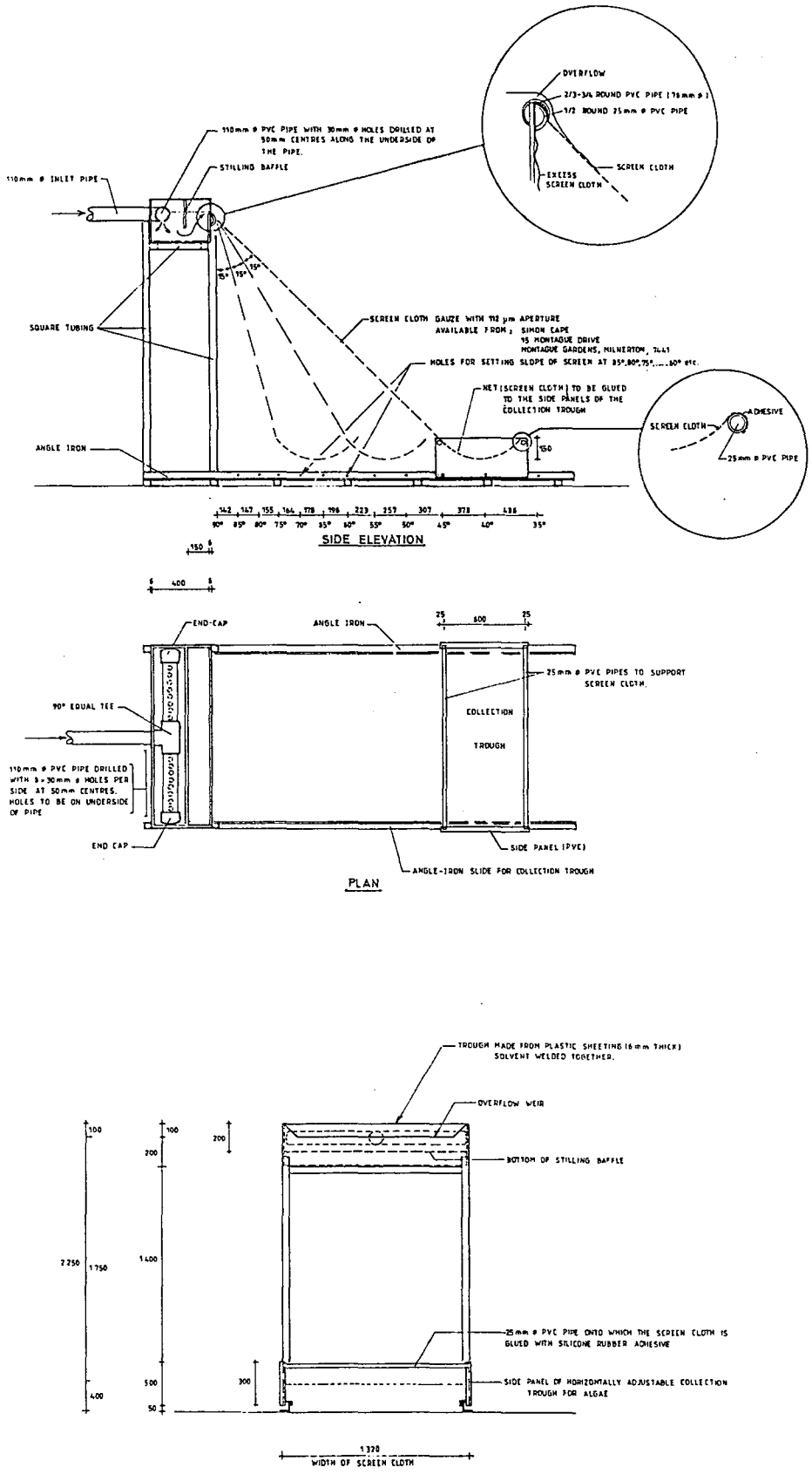

Figure 4.3 Side, plan and front elevation design of the technical-scale screen harvester for use at WTC, Wellington.

A series of holes were drilled in the angle iron at the base to allow for 15° changes in the collection trough angles.

A screen of 80 μm mesh size was used. The screen was secured at the top by clamping the upper edge between a $^3/_4$- and $^1/_2$-round PVC piping. This, along with the variable degrees of screen slope allowed by the angle iron, allowed for selection of the optimal tautness of the screen. The lateral edges of the screen were secured to the side panels of the collection trough by clamping between semi-elliptical cut-out sections which were bolted to the outer side panel. This prevented the cell concentrate from spilling over the sides.

PVC piping was used in the construction to prevent the corrosion under the high-salt conditions prevailing in the effluent.

The gross dimensions of this 2.25 m-high harvester are shown on Figure 4.3.

A technical-scale harvest of the biomass in pond D was then carried out using the scale-up screen harvester. The starting biomass slurry was skimmed from the surface floc. Figure 4.4 shows the harvester in operation at WTC.

Figure 4.4 Technical-scale harvest of the *Spirulina* biomass in pond D at WTC, Wellington.

4.3.3 Drying and Processing

Sun-drying beds were constructed from square-edged purlins, covered with chicken-wire mesh. The wire was covered by a layer of thick muslin. The concentrated cell slurry was spooned onto the muslin. Excess water drained out of the concentrate, and evaporative drying took care of the rest. The on-site sun-drying operation is shown in Figure 4.5. The biomass slurry dried into flat cakes approximately 0.5 cm thick.

The cakes were stripped from the muslin, and milled in an industrial mill to a coarse, green powder. All subsequent analyses were performed using this granular powder.

Figure 4.5 Sun-drying the *Spirulina* biomass on muslin beds.

4.4 RESULTS

The small-scale harvest of the autoflocculated biomass (2.2% solids) yielded a thick cell slurry of 21.4% solids concentration. Screen harvesting thus resulted in a 10-fold harvesting efficiency. Theoretical calculations based on *Spirulina* distribution and concentration in the entire water column, show that a near 100-fold concentration of biomass is possible if a

mechanical pumping device is used, utilising the water-column *Spirulina* instead of the autoflocculated biomass.

Sun-drying yielded 25 kg biomass (dry weight) in the form of thin cakes (approximately 5 mm thick).

The technical-scale model of the screen harvester yielded cell concentrations of the same magnitude as obtained with the small-scale harvester. Sun-drying of this biomass yielded 250 kg biomass (dry weight).

A 10-fold harvest scale-up evaluation was thus performed.

The resulting dried cakes were milled, and yielded a coarse powder with a moisture content of 22.7%.

4.5 DISCUSSION

The vast potential of microalgae could remain untapped were it not for an effective cell separation process. An effective process for the separation of *Spirulina* from tannery effluent is thus needed in order to reap the full economic benefits of this source of biomass.

Most processes of algal concentration require a preconcentration step for initial dewatering. Various chemical and physical flocculants are widely used for the preconcentration of unicellular forms. The harvest of *Spirulina* from the ponded effluent need not involve this step, as autoflocculation occurs by virtue of the gas vesicles in the cytoplasm of cell. Skimming of the surface floc yields a high enough solids fraction to proceed immediately with the screening step.

The small-scale harvest with the 100 μm mesh resulted in a ten-fold concentration of the *Spirulina* biomass, from 2.2% to 21.4% solids concentration (dry weight). Total harvested biomass amounted to 25 kg (dry weight).

Similar solids concentrations were achieved with the scale-up technical scale model, even though a mesh of 80 μm was used. The filaments were long enough to be entrapped by this screen. A biomass yield of 250 kg was obtained, thus providing a 10-times scale-up yield.

The harvested biomass was sun-dried on muslin beds without any post-harvest step. The effectiveness of this process of drying is discussed in the following chapter.

The experimental, small-scale and scale-up evaluation of the screen harvest has demonstrated that *Spirulina* cells can be successfully concentrated from the tannery effluent medium. These results indicate that an industrial-size, scale-up, automated model of the screen harvester is the next step in the optimization of this process. It is envisaged that the automated harvester would concentrate the water-column biomass, as apposed to the autoflocculated mat. The virtues of this are expounded upon in the following chapters. A design modification in the recovery of the cell slurry from the collecting reservoir is also necessary, with direct transfer to the drying racks. A conveyer-belt type mechanism would seem to be the most effective way of performing this.

The major potential problem associated with screen harvest of relatively large microalgae from the pond medium is the enrichment and subsequent contamination of the filtrate with smaller, unicellular forms. Use of a screen for the removal of larger organisms may result in smaller organisms passing through the large pores of the screen, and, coupled with organic enrichment associated with cellular damage during the harvesting process, may result in a species shift away from the organism of interest. The enrichment for *Chlorella* proliferation following *Spirulina* harvesting was noted by Richmond (1981). It is their hypothesis that continual harvest of the larger *Spirulina* resulted in constant augmentation of the smaller *Chlorella*. This could present a very real problem following the harvest of *Spirulina* from the tannery effluent, as the presence of the unicellular chlorophyte, *Dunaliella*, has been noted. A further investigation into the succession potential of unicellular chlorophytes following the cyanobacterial harvest needs to be undertaken, although initially this problem may be overcome by pumping the filtrate to one of the initial ponds. The chemical conditions in the initial ponds may not satisfy the growth requirements of the chlorophyte contaminants.

When the filtered effluent eventually reaches the latter ponds again, the chemical nature would be altered sufficiently to allow for continued proliferation of the cyanobacteria.

It is suggested by Richmond (1981) that the wet product obtained by screen harvesting could be used directly as animal or fish feed without any further dehydration. However, for the successful commercialization of the procedure, storage and transportation of the final product necessitates the complete dewatering of the biomass, so as to minimise transport and preservation costs. It has been suggested that dehydration may be successfully achieved by mixing the algal slurry with dry additives such as straw, sugar beet pulp, meal powder or grains, and thereafter pressing the mixture by extrusion, producing pellets instead of powder. Richmond (1981) succeeded in producing four different products which could be stored unrefridgerated for months. These included drum-dried algal flakes, canned slurry, sun-dried mixed algal pellets, and lyophilised cakes. The sun-dried pellets were prepared by mixing the 20% slurry with an equal weight of corn meal. Wet pellets were first formed by milling the mixture in a commercial meat grinder, and dried on polyethylene sheets in the sun for 1-2 days. Their final product contained less than 7% water.

Richmond (1981) postulates that sun-drying represents the only economically feasible processing step, and that, eventually, all the algal products produced at the Jacob Blaustein Institute for Desert Research will be processed using solar energy.

CHAPTER 5

CHEMICAL COMPOSITION

Summary An evaluation of the chemical composition of harvested *Spirulina* biomass was undertaken. The dried biomass was found to contain 48.5% protein, 14.9% carbohydrate, 7.6% crude lipid, 15.0% ash, and 22.7% moisture. Amino acid analysis revealed an expected deficiency in certain essential amino acids. Pigments included 2.9 g carotenoids.kg^{-1}, 1.7 g xanthophyll.kg^{-1}, 2.7 g chlorophyll$_a$.kg^{-1}, and 0.5 g phycobiliproteins.kg^{-1} dried biomass. The biomass had an energy content of 17.0 kJ.g^{-1} dry weight.

5.1 INTRODUCTION

The utilization of *Spirulina* as a protein source in human and animal rations has been well documented (Becker, 1986; Ciferri, 1983; de Pauw & Persoone, 1988; Becker, 1988). The interest in this blue-green algae has been stimulated by its seemingly ideal chemical composition for nutritional application (Richmond, 1988).

One of the first analyses of naturally occurring *Spirulina* revealed a protein content of 45% (dry wt) and 62% in laboratory-grown biomass (Ciferri, 1983). More recent analyses have shown a high protein content, 71% protein being measured in cultures from Lake Texcoco (Durand-Chastel, 1980). The observed variation in protein content stems mainly from the growth conditions and percentage of ash. When the biomass sample that is obtained by filtration is not sufficiently washed with acid water to remove adsorbed carbonates, the ash content may be as high as 25%, and the protein content may decline to 50% or less. In acid-washed *Spirulina*, the protein content is 60-65%, rarely reaching 70% (Richmond, 1988).

The protein content of *Spirulina* also appears to be high when compared with that of unicellular algae and other cyanobacteria, although protein contents of close to 60% have been reported in *Chlorella* and *Scenedesmus*.

Ciferri (1983) reports a comparison between laboratory and open-pond grown *Spirulina*, where it was found that, while the laboratory grown biomass exhibited higher protein contents, the biomass grown in open ponds possessed higher percentages of carbohydrates and ash. In addition, cultures grown in open ponds comprise more protein and less carbohydrate than those grown in polyethylene tubes.

The amino acid profile of *Spirulina* is similar to that of other microorganisms. When compared to well-balanced feed proteins such as eggs or milk, however, *Spirulina* is somewhat deficient in the essential amino acids methionine, cysteine, lysine and tryptophan. A considerable degree of variation exists in the amino acid profiles of *Spirulina* from different sources, with significant differences especially noted in the concentration of the essential sulphur amino acids. Ciferri (1983) concludes from all the reports that, although *Spirulina* may be deficient in certain amino acids, it is superior to all plant protein, including that from legumes.

Carbohydrates account for 15-20% of the dry weight (Ciferri, 1983). Hydrolysis of the carbohydrates yields glucose, levulose, sucrose, glycerol and several polyols. The main carbohydrate storage product is glucosan and rhamnosan, both containing glucosamine. Degradation of the cell wall yields glucosamine and muramic acid, which are associated with peptides rich in glycine, serine, alanine and glutamic acid (Richmond, 1988).

An evaluation of the vitamins in *Spirulina* has also been undertaken. It was found that cyanocobalamin appears to be abundant, with concentrations of 1.6% (Richmond, 1988) to 11 $mg.kg^{-1}$ (Santillan, 1982) of dried cells being reported. In fact, *Spirulina* appears to have the highest concentration of vitamin B_{12} content of any unprocessed plant or animal food. Other vitamins of note include β-carotene (provitamin A) (1700 $mg.kg^{-1}$), Ca-pantothenate (11 $mg.kg^{-1}$), inositol (350 $mg.kg^{-1}$), niacin (118 $mg.kg^{-1}$) and tocopherol (190 $mg.kg^{-1}$) (Richmond, 1988).

Considerable variations have been reported in the fatty acid content, with values ranging from 1.5-12% of the dry weight (Ciferri, 1983). Tornabene *et al.* (1985) report a high lipid content of 16.6% of dry weight, while, at the other end of the range, Switzer (1980, cited in

Richmond, 1988) found a lipid content of 5%. In both *S.platensis* and *S.maxima*, free fatty acids account for 70-80% of the total lipids, the remaining being chiefly mono- and digalactosyl glycerides and phosphatidyl glycerol.

A characteristic of *Spirulina* is its high concentrations of gamma-linolenic acid (GLA) (6,9,12-octadecatrienoic acid), which is synthesised by direct desaturation of linolenic acid (Ciferri, 1983), and linoleic acid. Concentrations have been estimated at 1.04 and 1.24%, respectively (Richmond, 1988). The occurrence of GLA is rare, and is not found in foods other than human milk (Cohen *et al.*, 1987). GLA seems to fulfil the role played by alpha-linolenic acid in algae and higher plants (Nichols & Wood, 1968), and this fatty acid has been found in 19 strains of *Spirulina* (Cohen *et al.*, 1987). The therapeutic properties of GLA include the lowering of the plasma cholesterol level and in the treatment of atopic eczema, premenstrual syndrome, heart diseases, Parkinson's disease, and multiple sclerosis (Cohen *et al.*, 1987). Cohen & Vonshak (1991) suggest that the fatty acid composition of *Spirulina* strains may be used in the chemotaxonomy of this group.

Poly-β-hydroxybutyrate has also been isolated from *S.platensis* (Campbell *et al.*, 1982). This compound acts as a carbon and energy reserve, and accumulates during the exponential phase of growth, reaching concentrations of 6% of the dry weight.

The only chlorophyll pigment present is chlorophyll $_a$, and accounts for 0.8-1.5% of the dry weight. Mixoxanthophyll and β-carotene are the major carotenoids, their concentrations ranging from 0.2-0.4% of the dry weight (Ciferri, 1983). Tornabene *et al.* (1985) report a total carotenoid and xanthophyll content of 0.5% of the organic weight. The fairly high concentration of these pigments has stimulated the interest in the use of this organism as a source of pigments for fish, chickens and eggs.

The phycobiliproteins, c-phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE), comprise about 20% of the cellular protein, and are quantitatively the dominant pigments in *Spirulina* (Richmond, 1988). c-Phycocyanin is present in all cyanobacteria (Boussiba & Richmond, 1980; Stanier & Cohen- Bazire, 1977), and its concentration depends on environmental conditions (Myers & Katz, 1955; Bryant, 1981). There is evidence that c-

phycocyanin serves as a nitrogen storage compound in *Spirulina* (Boussiba & Richmond, 1980). Phycocyanin and phycoerythrin could be used as natural pigments for the food, drug and cosmetic industry to replace the currently used synthetic pigments which are suspected of being carcinogens. Phycocyanin from *Spirulina* has already been commercialised by Dainippon Ink & Chemicals of Japan under the name of Linablue. Its uses include colouring of candy, ice-cream, dairy products and soft drinks. A patent has been procured by Dainippon for the buffer extraction of phycocyanin, and the treatment of the extract with an organic solvent, which denatures and precipitates the phycocyanin. The blue pigment obtained is used in eye shadow, eye-liner and lipstick (Cohen, 1986).

5.2 RESEARCH OBJECTIVES

The logical step following harvesting and drying is that of chemical evaluation of the final product, in terms of its intended use, and in order to determine whether the effluent medium, harvesting and drying process affects the product in any way. The primary aim of this facet is the comparison between the published chemical composition of pure-culture grown biomass, and this effluent-source of *Spirulina*. The following analyses were thus highlighted.

1. Protein content
2. Amino-acid profile
3. Carbohydrate content
4. Crude lipid content
5. Carotenoids and Xanthophylls
6. Phycobiliproteins
7. Chlorophylls
8. Ash content
9. Moisture content
10. Energy value
11. Physical properties

5.3 MATERIALS AND METHODS

5.3.1 Total Kjeldahl Nitrogen (TKN)

The method used followed that of APHA (1980). A dried sample of *Spirulina* biomass was placed in a 500 ml Kjeldahl flask containing a few glass beads, and digested with 100 ml digestion mixture using a kjeldahl apparatus for the suitable removal of the SO_3 fumes. Following complete oxidation of the sample (indicated by a cessation of SO_3 production) the sample was boiled for a further 30 mins. The resulting solution was cooled, and to this was added 200 ml H_20, a small quantity of Carborundum powder, and dechlorinating agent at a ratio of 1 ml per 100 mg Cl^- ions. The solution was cooled, and 100 ml 12 N NaOH was then added. The sample was immediately distilled, and the distillate (200 ml) was collected below the surface of indicating boric acid (50 ml). The ammonia in the distillate was titrated with 0.2 N H_2SO_4. The end-point was reached when the solution turned from green to lavender.

TKN was calculated as follows:

$$TKN\ (mg/l) = \frac{A \times N \times 14000}{\text{sample volume (ml)}}$$

where A = volume (ml) 0.2 N H_2SO_4

N = normality of the H_2SO_4

The value obtained was converted to %, and total protein was calculated by multiplication by 6.25.

5.3.2 Amino-Acids

The amino acids were analysed according to the gas-liquid chromatographic protocol suggested by Beckman in the System 6300 Application notes, and was carried out in conjunction with the University of Natal, Animal and Poultry Science Laboratory.

Sample preparation involved acid hydrolysis using 6 N HCl at 110°C for 24 hrs. As suggested by Keutman & Potts (1969), mercaptoethanol was added as an antioxidant to the HCl to protect the samples during hydrolysis.

Retention times and calibration factors were derived using the high-purity hydrolyzate amino acid standard (STD) produced by Beckman. Working solutions of the 2.5 μm/ml/component solution were prepared by dilution with Na-S Sample Dilution Buffer.

Norleucine was used as an internal standard to correct for partial injections and volume abberations. Norleucine was chosen because of its resistance to the rigors of acid hydrolysis. It elutes in the peak between leucine and tyrosine. A 2.50 μm/ml solution of norleucine in Na-S sample dilution buffer was incorporated in both the calibration sample and the biomass sample.

Four buffer changes were necessary for satisfactory elution, and included Na-R, Na-A, Na-B, and Na-D Beckman buffers. Buffer change times were adjusted first to position the Na-A to Na-B buffer change artifact between valine and methionine, and the Na-B to Na-D artifact between phenylalanine and histidine.

Three step-wise temperature changes were needed: 50.0°C (44 mins), 65.0°C (2 mins), and 70.0°C (15.5 mins).

A flow rate of 20 ml/hr was maintained throughout the entire procedure.

This procedure also proved suitable for the quantification of ammonia.

5.3.3 Carbohydrate Content

Total carbohydrates were determined according to the Phenol- Reaction method of Gerhardt *et al.* (1981). A standard solution was prepared by dissolving 100 mg of glucose in 100 ml 0.15% (w/v) benzoic acid, which acts as a preserving agent. The solution was then diluted

1:10 in distilled water just prior to use, to give a solution containing 100 μg/ml. A range of standard solutions were then prepared from 0-100 μg/ml.

Duplicate suspensions of 100 mg/ml *Spirulina* biomass were them prepared in distilled water. Duplicate dilutions were then prepared in distilled water, and contained 0.5 and 0.25 mg/ml biomass.

One ml of each of the standards and the *Spirulina* suspension were pipetted into test-tubes. The blank contained 1 ml dH_2O. One ml phenol reagent (5% w/v) was added to each tube, and mixed rapidly. Five ml concentrated H_2SO_4 was added to each tube, and mixed rapidly. The tubes were allowed to stand at room temperature for 10 mins. The tubes were then placed in a 25°C water bath for 15 min, and the absorbances read at 488 nm.

A standard curve was prepared, plotting standard concentrations against absorbance. The concentrations of the hydrolysed *Spirulina* biomass was extrapolated from the linear equation.

5.3.4 Crude Total Lipid Content

The method outlined by Gerhardt *et al.* (1981) was used for the determination of crude total lipids. Duplicate suspensions of *Spirulina* biomass in the range 0.250-1.005 g dried biomass were prepared in 4 ml dH_2O in glass-stoppered vials. To this was added 5 ml chloroform and 10 ml absolute methanol. The mixture was shaken for 5 mins, and then stood at room temperature for 3 hrs with intermittent shaking. Another 5 ml chloroform and 5 ml dH_2O was added, and the mixture was shaken at room temperature for 30 mins.

The extract was filtered through Whatman no. 1 filter paper. The filtrate was transferred to a glass cylinder, and the phases were allowed to separate. The methanol-water layer was removed.

The residue of cell material that remained on the filter paper was then re-extracted by shaking with 5 ml chloroform. The mixture was re-filtered, and the filter rinsed with 12.5 ml

chloroform. The two filtrates were combined in a weighed porcelain container, and evaporated to dryness under a stream of nitrogen in a 40°C water bath.

The container was re-weighed, and the dry crude total lipid content could be determined by subtraction.

5.3.5 Carotenoids and Xanthophylls

Carotenoids and xanthophylls were extracted by resuspending 250 mg *Spirulina* biomass in ice-cold acetone (-20°C) containing 2,6-Di-*tert*-butyl-*p*-cresol (butylated hydroxytoluene, 20 mg/l) added as an antioxidant. The solution was sonicated and centrifuged three times. The supernatants were then pooled, and dried to under vacuum at 30°C. The residue was re-dissolved in 70% aqueous methanol and passed through a prerinsed Sep-Pak C_{18} cartridge (Waters Chromatography Division, Millipore Corp., Milford, MA) and the pigments were eluted with 1.0 ml methylene chloride. Aliquots were analyzed by reverse-phase HPLC. The procedure was performed in dim light to minimise photo-oxidation and isomerisation of the compounds.

The HPLC system employed a Nucleosil 5 μm C_{18} (250 x 4.6 mm i.d.) column (Macherey-Nagel, Duren, West Germany) with a linear gradient of 0-100% ethyl acetate in acetonitrile/water (9:1, v/v), containing 0.1% (v/v) triethylamine. Flow rate was maintained at 0.8 ml/min for 25 min. Pigment peaks were detected at 410 nm using a LINEAR UVIS 2000 variable wavelength detector, and quantified by peak integration (Spectra-Physics SP 4290 integrator) after calibration with ß-carotene (Sigma). The concentration of each compound was derived from a ratio conversion using the β-carotene standard. Normalised coefficients were derived for each compound of interest, as described by de las Rivas *et al.* (1989), and used for the calculation of corrected concentration values. Normalised coefficients used were : xanthophylls (1.41-1.71, depending on the retention time), lutein (1.41), zeaxanthin (1.34) and β-carotene (1.00).

5.3.6 Phycobiliprotein Estimation

The procedure for the quantification of the phycobiliproteins was essentially that of Bennet & Bogorad (1973). Milled *Spirulina* biomass was resuspended in 0.01 M Na-Phosphate buffer (pH 7.0). The suspension was sonicated for 15 sec pulses for 1 min, and centrifuged at 81 000 g (1 hr). The supernatant was analyzed spectrophotometrically for PC, APC and PE.

The relative amounts of the three phycobiliproteins were calculated according the formulae prescribed in Haury (1980):

$$PC = \frac{A_{615} - 0.474\ (A_{652})}{5.34}$$

$$APC = \frac{A_{652} - 0.208\ (A_{615})}{5.09}$$

$$PE = \frac{A_{562} - 2.41\ (PC) - 0.849\ (APC)}{9.62}$$

The Beer-Lambert law (A = *E*cl) was used for the calculation of the absolute concentrations of PC and APC, using the extinction coefficients prescribed by Richmond (1981):

$E^{1\%}_{1cm}$ for PC = 73 (at 620 nm), and
$E^{1\%}_{1cm}$ for APC = 58 (at 580 nm)

The absolute concentration of PE was estimated using the relative concentration value of PE in proportion to the absolute value of PC.

Results were calculated as:

Relative values
% Dry Weight
$g.kg^{-1}$ Dry Weight
% Pigment/Protein

5.3.7 Chlorophyll Content

The pellet left after buffer extraction of the phycobiliproteins was re-extracted in 80% acetone (v/v), and the absorbance determined at 664 and 647 nm, in order to estimate the chlorophyll concentration. The formulas suggested by Lichtenthaler (1987) were used for the quantification of chlorophyll a and b, where:

$$Chlorophyll_a = 12.25\, A_{\underline{664}} - 2.97\, A_{\underline{647}}$$
$$Chlorophyll_b = 21.50\, A_{\underline{647}} - 5.10\, A_{\underline{664}}$$

$Chlorophyll_b$ concentration was estimated so as to get an approximation of the degree of chlorophyte contamination of the *Spirulina* biomass.

5.2.8 Ash Content

Ash determination was performed in triplicate, with biomass weights ranging from 1.012-3.020 g. Porcelain crucibles were heated at 100°C overnight, dried in a desiccator (2 hrs), and weighed. Three dried biomass samples (60°C/3 hrs) of known weights were added to the crucibles and ashed (600°C/24 hrs), burning off all the organics. Crucibles were again placed in a desiccator (2 hrs), and reweighed. The difference between the starting biomass weight and the ashed weight gave the ash content of the different samples.

5.3.9 Moisture Content

Triplicate quantities of milled *Spirulina* were weighed into pre-dried and pre-weighed crucibles, and dried at 105°C until no further weight loss was observed (approximately 5 hrs). The moisture content was then determined by subtraction.

5.3.10 Energy Determination

The amount of energy present in the *Spirulina* biomass was determined using a dds CP400 bomb calorimeter. Benzoic acid was used as a calibration standard. Gelatin capsules of known energy values were filled with milled *Spirulina*, and fired in the calorimeter. Each determination required a temperature equilibration of 8 mins, and a reading stabilization of 4 mins.

5.3.11 Physical Properties

The *Spirulina* biomass was also evaluated in terms of appearance, colour, odour, taste and particle size.

5.4 RESULTS

5.4.1 Total Kjeldahl Nitrogen

TKN calculated as N measured 7.76%. Total protein thus amounts to:

$$7.76 \times 6.25 = 48.5\% \text{ Protein}$$

5.4.2 Amino Acids

The concentrations of the amino acids in the hydrolysate are shown in Table 5.1. The order of the compounds in Table 5.1 represents the elution order. The concentration of ammonia

is also shown at its relative elution time. Norleucine, used as an internal standard, elutes between Leucine and Tyrosine.

Table 5.1 Amino Acid concentration of dried *Spirulina* biomass.

Amino Acid	% of Protein	% Dry Weight
Aspartate	4.695	2.104
Threonine	2.667	1.195
Serine	2.886	1.293
Glutamine	6.559	2.939
Proline	2.185	0.979
Glycine	4.401	1.972
Alanine	5.269	2.361
Cystine		
Valine	3.684	1.651
Methionine	0.968	0.434
Isoleucine	2.946	1.320
Leucine	4.483	2.009
Norleucine (I.S)		
Tyrosine	1.417	0.635
Phenylalanine	1.767	0.792
Histidine	0.685	0.307
Lysine	2.270	1.017
Ammonia	5.967	2.674
Arginine	2.455	1.100

The first column (% of protein) indicates that some loss of amino acids does occur during sample preparation, although the complete elution of the Norleucine standard does not show this due to its stability during sample preparation.

5.4.3 Carbohydrate Content

The standard curve yielded a linear equation of $x = {}^{y}/_{7.656}$. This formula was used for the calculation of the carbohydrate content of the *Spirulina* biomass. The mean value of 6 determinations yielded a carbohydrate content of 14.9% of the dry weight (SD = 1.3).

5.4.4 Crude Total Lipid Content

The mean of 6 determinations yielded a crude total lipid content of 7.614% of the dry weight (SD = 0.907).

5.4.5 Carotenoids and Xanthophylls

The HPLC of the solvent-extracted pigments yielded the following carotenoid and xanthophyll concentrations:

Table 5.2 Pigment concentrations in the *Spirulina* biomass as determined by reverse-phase HPLC.

Pigment	% Dry Weight	$g.kg^{-1}$ Dry Weight
Unidentified Xanthophylls	0.001	0.007
	0.145	1.448
	0.022	0.224
lutein	0.150	1.496
β-carotene	0.141	1.408

Total xanthophylls amount to 1.679 g/kg (dry wt). Total carotenoids (β-carotene + lutein) amount to 2.904 g/kg (dry wt).

5.4.6 Phycobiliprotein Estimation

The analysis of the three phycobiliproteins (PBP) is shown in Table 5.3.

Table 5.3 Concentration analyses of PC, APC and PE in the *Spirulina* biomass.

PBP	Relative Amount	% Dry Weight	$g.kg^{-1}$ Biomass	% PBP of Protein
PC	0.016	0.022	0.225	0.046
APC	0.011	0.020	0.201	0.041
PE	0.002	0.003	0.025	0.005
TOTAL		0.045	0.451	0.092

5.4.7 Chlorophyll Determination

The concentrations of the chlorophylls in the acetone extract of *Spirulina* are as follows:

$Chlorophyll_a$ = 2.692 g/kg (dry wt)
$Chlorophyll_b$ = 1.205 g/kg (dry wt)

5.4.8 Ash Content

Triplicate determinations of ash content revealed an ash content of 15.04% of the dry weight (SD = 0.26), which gives an indication of the inorganic salts and compounds present in the *Spirulina* biomass.

5.4.9 Moisture Content

The mean of triplicate determinations of moisture yielded a moisture content of 22.7% (SD = 0.4).

5.4.10 Energy Content

The mean of 5 separate calorimetric energy determinations yielded an energy value of 17.008 $kJ.g^{-1}$ dry weight (SD = 0.051).

5.3.11 Physical Properties

The *Spirulina* biomass had the following physical characteristics:

Appearance	Fine powder
Colour	Dark blue-green
Odour and Taste	Salty, sea-weed like
Particle Size	400-500 μm

5.4 DISCUSSION

The protein content (48.5%) is low in comparison to those found by previous authors: 60-71% (Durand-Chastell, 1980), 60% (Tel-Or *et al.*, 1980), 71% (Richmond, 1988) and 60-70% (Earthrise Farms, California). The biomass used for harvesting originated from the surface floc in the effluent. Protein degradation had probably set in due to the extreme environmental conditions of light and temperature, especially significant at the surface, where the biomass is continually exposed to these conditions. Cell rupture may thus lead to loss of protein to the effluent. Estimations of protein content by other authors centres on pure-culture grown biomass, and it is known that differing environmental and medium conditions significantly affect the protein content.

The sun-drying method employed in this experiment (because of its ease and low cost of operation) is also known to lead to degradation of the protein, due to the length of time required to dewater the biomass. Although the lack of a cellulose cell wall seems to favour the sun-drying of *Spirulina* without any loss of digestibility (Venkataraman *et al.*, 1980), the protein-loss factor seems to override this advantage.

The relatively low protein content of harvested *Spirulina* biomass is concomitant with the relatively high ash content (15.04%), when compared to *Spirulina* biomass from pure-culture grown biomass: 9% (Richmond 1986b), 7-13% (Earthrise Farms, California) and 6.4-9% (Durand-Chastel, 1980). This is probably as a result of the failure to wash the concentrated cells with acid water (Richmond, 1988). The ash value represents the inorganic content, and includes adsorbed and absorbed salts and minerals. The ash content depends primarily on the composition of the medium. Microalgae usually contain less than 10% of their dry weight as ash, and the ash content only marginally affects the nutritional quality of the biomass (Becker, 1986). The high ash content seems to be an area of concern, however, when considering the effluent-source of the medium. The ash content is contributed to by unused minerals from the culture medium, and may contain a variety of toxic inorganic minerals or compounds. It is also known that a high concentration of unused minerals results in a change in the proportion of the other major cellular constituents (Becker, 1986).

An analysis of the amino acid content was undertaken, as the nutritive quality of the proteins can be estimated from the content, proportion and availability of its amino acids. Of special interest are the essential amino acids, which animals are incapable of synthesising. The essential amino acids, elucidated with the aid of feeding studies, include isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Arginine and histidine are considered semi-essential, as they have to be supplied exogenously during growth (Becker, 1986). Animal species differ quantitatively and qualitatively in their amino acid requirements. Because of this, the availability of the different amino acids in the *Spirulina* biomass will be discussed in relation to their nutritive value in the various aquaculture organisms used in feeding trials. These are detailed in Chapters 7 & 8.

Unfortunately, the GLC method employed (and, indeed, all methods currently in use) fail to differentiate between the total amount and the degree of availability of the amino acids (Becker, 1986). This is of special importance in lysine and methionine, where prolonged storage or heat treatment of the protein results in the free *E*-amino group of lysine compounding with reducing carbohydrates, making the lysine unavailable for digestion (Becker, 1986).

In order to compare the amino acid content of tannery effluent-grown *Spirulina* to those obtained by other authors, and the levels suggested by the Food Administration Organisation (FAO), the amino acid content shown in Table 5.1 was converted to g amino acid/16 g N, the conversion taking into account the TKN value of the biomass.

Table 5.4 Comparison of amino acid patterns of *Spirulina* from various sources (g amino acid/16 g N)

Amino Acid	FAO	*Effluent Grown Spir.sp.*	*S.maxima*	*S.plat.*	*Spir.sp.*
Ile	4.0	3.0	6.0	6.7	4.8
Leu	7.0	4.5	8.0	9.8	8.4
Val	5.0	5.0	6.5	7.1	5.4
Lys	5.5	2.3	4.6	4.8	4.7
Phe	6.0	1.8	4.9	5.3	4.0
Tyr		1.4	3.9	5.3	
Met	3.5	1.0	1.4	2.5	2.3
Cys			0.4	0.9	1.0
Try	1.0		1.4	0.3	1.5
Thr	4.0	2.9	4.6	6.2	4.6
Ala		5.3	6.8	9.5	6.9
Arg		2.5	6.5	7.3	6.6
Asp		4.7	8.6	11.8	9.1
Glu		6.6	12.6	10.3	12.2
Gly		4.4	4.8	5.7	4.9
His		0.7	1.8	2.2	1.6
Pro		2.2	3.9	4.2	3.8
Ser		2.9	4.2	5.1	4.9
Ref	Becker, 1986	This Study	Clement *et al.*, 1967	Becker & Venkataraman, 1984	Waltz, 1982

When compared to the levels suggested by the FAO, this source of effluent-grown *Spirulina* appears to be deficient in a number of essential amino acids, including isoleucine (25%

deficiency), leucine (36%), lysine (60%), phenylalanine (72%), methionine (71%) and threonine (33%). The other *Spirulina* species included for comparison are also deficient, to varying degrees in the essential sulphur amino acids, especially lysine, phenylalanine and methionine.

This deficiency in amino acids almost surely arises from the fact that the surface flocculated mat was used for harvesting. Protein degradation and loss to the effluent medium probably resulted due to the extreme environmental conditions prevailing at the surface. Loss may also have occurred due to the lengthy drying time associated with sun-drying and may have been caused by leaching out in the gravity filtered medium, and/or due to bacterial activity associated with the lengthy drying period.

The main practical consideration in minimising protein loss is thus to investigate a faster, more efficient drying procedure.

The carbohydrate content of the *Spirulina* biomass (14.9%) is comparable to that of other sources: 16.5% (Richmond, 1986b), 16% (Durand-Chastel, 1980), 8-14% (Tipnis & Pratt, 1960), 15-25% (Earthrise Farms, California) and 17% (Becker & Venkataraman, 1984).

The lipid content (7.614%) is comparable to that of other sources: 6.0-7.0% (Durand-Chastel, 1980), 4-9% (Tipnins & Pratt, 1960), 4-7% (Earthrise Farms, California) and 3.0% (Becker & Venkataraman, 1984).

The total xanthophyll content (1.679 $g.kg^{-1}$) is comparable to that of other sources of *Spirulina*: 1.80 $g.kg^{-1}$ (Richmond, 1986b), 1.40-1.80 $g.kg^{-1}$ (Durand-Chastel, 1980).

The chlorophyll$_a$ content of the dried *Spirulina* is 2.692 $g.kg^{-1}$ dried biomass. This is generally lower than the levels found by other authors: 6.10-7.60 $g.kg^{-1}$ (Durand-Chastel, 1980) and 11 $g.kg^{-1}$ (Earthrise Farms). There is also a chlorophyll$_b$ content of 1.205 $g.kg^{-1}$. Because cyanobacteria, including *Spirulina*, only contains chlorophyll$_a$, the presence of chlorophyll$_b$ indicates contamination of the surface floc by other green algae. This, in part,

may help to explain the comparatively lower protein and amino acid content of the harvested biomass.

Total phycobiliproteins in the dried *Spirulina* biomass amount to 0.451 $g.kg^{-1}$ dry weight. This is substantially lower than the levels found by Earthrise Farms (150 $g.kg^{-1}$), and suggested by Tel-Or *et al.* (1980), and should range between 10-30 $g.kg^{-1}$. This, again, can be explained by the severe environmental conditions prevailing at the surface, which may lead to degradation of the light-sensitive phycobiliproteins. It is also known that the levels of phycobiliproteins fluctuate with the prevailing environmental conditions (Richmond, 1986b), especially in response to various lighting regimes. The variable light-conditions in the effluent ponds caused by the continual shifts in vertical distribution of microbial populations may contribute to the phycobiliprotein content, but this aspect will need to be looked at in more detail in order to maximise the amount of these light-harvesting pigments.

Quantitatively, the low amount of phycobiliproteins present in the *Spirulina* biomass does not correspond with the relatively high carotenoid levels (2.904 $g.kg^{-1}$ dry weight), which is of a magnitude higher than those quoted by other authors: 1.90 $g.kg^{-1}$ (Richmond, 1986b), 1.50-1.90 $g.kg^{-1}$ (Durand-Chastel, 1980). This may be due to the fact that the *Spirulina* bloom traps cells at the surface of the pond, and subjects them to the danger of photodynamic stress. Healthy cells may thus counteract this danger by an increase in cellular carotenoid levels which screen out much of the harmful UV irradiation. This phenomenon was observed by Paerl *et al.* (1983) with the cyanobacterium *Microcystis aeruginosa*. This may also explain the lower levels of chlorophylls observed, which is usually concomitant with higher levels of carotenoids and lower levels of phycobiliproteins.

CHAPTER 6

TOXICOLOGICAL EVALUATION OF *Spirulina* BIOMASS

Summary The toxicological properties of dried and milled tannery-effluent generated *Spirulina* were evaluated. Analyses included nucleic acid, pesticide and heavy metal contents. A bioassay was performed using *Artemia salina*. The biomass was used in a feeding trial with chickens, and intensive toxicological and pathological evaluations were performed. The analyses of the biomass and results of the feeding trial allows a preliminary conclusion that *Spirulina* has no decisive toxicological constraints.

6.1 INTRODUCTION

Algae destined for direct or indirect human consumption must be grown with the greatest attention to cleanliness and purity. Enclosed cultures thus far seem to be the most promising, although the high cost of establishing and maintaining such cultures present an economic problem (Oswald, 1980). *Spirulina* mass-culture seems to fit the criteria of low cost production and processing. The elevated growth of this organism at high temperatures and alkalinities is complemented by the concomitant decrease in predator growth and prevention of nitrate reduction at these high pH's.

At first, early studies by botanists suggested that the production of microalgae seemed a relatively simple process. Subsequent experience has revealed that it is a technically demanding enterprise, requiring an interdisciplinary approach. This has resulted in a complex, state-of-the-art marriage between engineering, technical optimization, economic feasibility studies, chemical evaluation, and nutritional studies (Soeder, 1980). The next biggest hurdle for broader application of microalgae has proved to be that of toxicological testing. Until now, there has been no government funding for research into this aspect (Soeder, 1980), in itself an intensive and extensive part of the science and feasibility of growing mass-cultures of algae destined for human or animal consumption.

The evaluation of the toxicological properties presents one of the greatest problems in the assessment of the suitability of any new feed source. Dried algal, bacterial, or fungal cells (van der Wal, 1980), when destined for food or feed-applications, are collectively referred to as "single-cell proteins" (SCP) (Litchfield, 1979).

Thus far, three main criteria for the evaluation of a SCP product have been noted :

1. Nutritional value of SCP products for use in human food or animal feeds.
2. Safety of SCP products for human and animal feeding.
3. Production of functional protein concentrates and isolates free from nucleic acids and toxic factors (Litchfield, 1979).

This chapter will concern itself primarily with the evaluation and discussion of toxic compounds potentially present in the biomass. There are several key problem areas in using SCP products for direct or indirect consumption by humans. These include mainly inherent toxic compounds and foreign-compound contamination.

The Protein Advisory Group (PAG) of the United Nations has developed guidelines for the preclinical testing of novel protein sources and supplementary foods (Litchfield, 1979).

Algal biomass grown under controlled, pure-culture conditions usually have a specified chemical content, with little or no threat of contamination from external sources. Growth in relatively variable tannery effluent presents theoretically uncontrollable conditions, with species specificity guaranteed only by the prevailing chemical composition of the effluent medium. Adsorption and uptake of chemicals by *Spirulina* from the effluent presents a source of exposure for this potential feed source to environmental chemicals. These non-intentional adulterants in food may also come from a variety of sources during the production, processing, packaging and storage of the biomass, as listed by Guzelian (1990) (Table 6.1).

Table 6.1 Sources of non-intentional food adulterants (after Guzelian, 1990)

During Production
Animal and insect filth Antibiotics Other agents for disease prevention and control Growth-promoting substances Microorganisms of toxicologic significance Parasitic organisms Pesticide residues Toxic metals and metallic compounds Radioactive compounds
During Processing
Animal and insect filth Microorganisms and their toxic metabolites Processing residues and miscellaneous foreign objects Radioactive compounds
During Packaging and Storage
Animal and insect filth Labelling and stamping materials Microorganisms and their toxic metabolites Migrants from packaging materials Toxic chemicals from external sources

6.1.1 Nucleic-acid Toxicosis

A major limitation to the use of microorganisms as a feed source is their nucleic acid content. The catabolism of the purine portion of nucleic acid yields, as its end product, uric acid. This compound is only slightly soluble in the human body fluids, and there is a risk that salts may be deposited in the renal tract or joints, leading to renal stones and/or gout. Ingestion of large quantities of algae might also produce elevated blood uric acid concentrations (Becker, 1980).

Gastrointestinal disturbances including nausea and vomiting, have been encountered in human feeding trials with a range of microalgae. These physiological symptoms seem to be caused by nucleic acid toxicosis. The symptoms have been observed by researchers working on algal feeding trials with humans (Dam *et al.*, 1965) as well as with feeding trials utilizing bacteria, yeasts and moulds as the protein source (Litchfield, 1979).

Published values of nucleic acid content of algae are in the range of 4-6% (Litchfield, 1979), with the *Spirulina* range of total nucleic acids being 2.9-4.5% in *Spirulina maxima* produced at Sosa Texcoco in Mexico (Durand-Chastel, 1980), 4% in *Spirulina* sp. (Aaronson *et al.*, 1980) and 2-5% in *Spirulina platensis* (Tipnis & Pratt, 1960). The nucleic acid content of microalgae, and in particular, *Spirulina*, compares favourably with that from other SCP sources: bacteria (up to 16%), yeasts (6-11%) and moulds (2.6-6.0%) (Litchfield, 1979).

Scrimshaw (1975, cited in Litchfield, 1979) suggests that a human intake of 2 g/day of yeast nucleic acid would be within safe limits. A significant risk of kidney stone formation or gout exists with intakes greater than 3 g/day. These guidelines are reiterated by Becker (1980), who also suggests that, since the nucleic acid content of algae varies, even from batch-to-batch, and, to leave a margin for safety, a maximum daily intake for humans of 30 g/day dried algae is recommended.

6.1.2 Pesticide Accumulation

The WTC effluent ponding system is situated in an area of intense agricultural activity, necessitating the evaluation of the pesticide content of the biomass. The possibility existed that pesticides may have accumulated due to run-off from the surrounding agricultural land.

There are three main groups of pesticides currently in use today. These include the organochloride, organophosphate and synthetic pyrethroid groups (SABS, pers comm.). Organochloride pesticides are extremely resistant to natural processes of decay in the environment, and may persist in the soil and water for decades after their production. Moreover, many of these agents are poorly excreted from living systems, and hence they progressively accumulate in plants and animals as they advance through the food chain. For example, although DDT (1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane) is present in air and rainwater in less than 1 part per billion (ppb), this chemical can be found in concentrations of 50 ppb in plants, 500 ppb in aquatic animals, 1000 ppb in mammals and birds, and greater than 5000 ppb in the body fat of humans (Guzelian, 1990).

With the improvement of techniques for residue analysis, the National Academy of Science Pesticide Residues Committee has urged that the criteria of no-residue and zero-tolerance levels be abandoned, and the criteria for negligible or permissible residues be implemented. Control measures with guidelines for human pesticide exposure have consequently been established by the Food and Agricultural Organisation of the United Nations (FAO) and the World Health Organisation (WHO) (Gudzinowicz, 1967). Yannai *et al.* (1980) tested wastewater-grown *Scenedesmus*, *Micractinium* and *Chlorella* for a range of pesticides. All tested biomass showed very low pesticide concentrations, except the total polychlorinated biphenyl (PCB) value, which exceeded 0.5 ppm, the legal limit in many countries. However, since the recommended limit for dried algae in animal rations is 10%, even extreme cases of feeding would result in a potential pesticide content far less than the legal limit.

The short- and long-term effects of pesticides on biological systems is continuously being investigated. The relationship between the metabolic deposition of chemicals and their toxicity has been elucidated, with research focusing on the liver as the major organ for the biotransformation of xenobiotics.

Most of the pesticide residues are lipophilic, and are consequently not readily excreted in aqueous media such as urine or bile. Were it not for metabolism, many of these chemicals would remain in the body indefinitely. Key enzymes involved are the cytochromes P-450, a collective term for a group of substrate-inducible hemoproteins that bind foreign chemicals and catalyse their oxidation to more polar derivatives, which are then easily excreted. Unfortunately, by the same token, oxidative activation of toxic compounds also occurs via the P-450-dependent enzymes in the liver or extrahepatic tissues. The potential detrimental effects of the metabolically activated toxins are counteracted by an array of conjugating enzymes located throughout the cell. These enzymes catalyse the conversion of oxygenated metabolites to even more water-soluble derivatives through the addition of polar ligands. In most instances, the conjugated products are inactive with respect to pharmacologic or toxic effects (Guzelian, 1990).

Extremely hydrophobic chemicals are hydrolysed only minimally, and are excreted from the body only very slowly, if at all. Examples are the organochloride pesticides (DDT, chlordane, dieldrin, Kepone), PCB's, and dioxins. Because the excretion of these chemicals is minimal, they accumulate in tissues and membrane lipids throughout the body (Guzelian, 1990).

Many organophosphate pesticides also share the properties of high lipid solubility and extensive distribution and storage in body fat. They, too, are poorly metabolised, with a resulting long total body half-life (Snodgrass, 1990).

6.1.3 Mineral & Heavy Metal Accumulation

Accumulation of heavy metals by algae, especially those destined as food or feed-sources, has also been well-documented. Concern is expressed in this field of research, reinforced by the exhaustive field of literature available documenting the toxicologic effects of heavy metal ingestion by humans (reviewed by Haddad & Winchester, 1990).

Yannai *et al.* (1980) tested wastewater-grown *Micractinium* and *Chlorella* for heavy metal accumulation and pesticide content, and used the algal powder in a range of feeding trials with chickens, carp, and in a secondary toxicity test with weanling rats. Although they found that the dried algal biomass was relatively rich in heavy metals (mercury, copper, cadmium, lead, aluminium and arsenic), there was no appreciable accumulation of any of these elements in the chickens' and carps' tissue examined, in spite of the fairly high concentration in the rations. In addition, no ill effects were noticed in the organs of the secondary test animals. Yannai and his co-workers (1980) suggest that the reason that the high concentration of the above elements failed to bring about corresponding high levels in the animal tissues, is due to the large amount of phosphorus present in the algae. The phosphorus seemed to be bound in the phosphate form, and all the above-mentioned metals forms water-insoluble salts with phosphate, rendering the metals unabsorbable from the gastrointestinal tract. The only exception was the slight accumulation of arsenic by the liver, but this, still, was below the limit suggested by the WHO.

Becker & Venkataraman (1980) have found that accumulation of mercury and cadmium by the alga *Scenedesmus* is unavoidable, as 80% of the final accumulated amount is reached after 24 hrs. Thus the only way to control the amount of metals taken up by the algae is to control the amount of heavy metals in the medium. As a post-harvest step, they proposed the washing of the algal biomass with 0.01 M EDTA (pH 8), which reduced the concentration of accumulated Hg and Cd by 90%.

Mercury related toxic effects in humans have been well documented (Aronow, 1990). Elemental and methyl mercury cross the blood-brain barrier and are distributed in the central nervous system. Target organ and tissues for mercury accumulation include the liver, spleen, bone-marrow, erythrocytes, intestine, skin and respiratory mucosa. It is excreted mostly through the kidneys (via glomerular filtration and tubular secretion) and the gastrointestinal tract.

Cadmium exposure in prolonged or acute doses may result in renal tubular dysfunction (Hall and Robertson, 1990).

Iron is absorbed into the mucosal cells of the duodenum and jejunum in the ferrous form, where it is oxidised to its ferric state, and then complexed to ferritin. Any additional iron is stored in the liver or spleen as ferritin. Because human excretion of iron is limited to 2 mg/day, in the overdose state, excess iron accumulates in the target organs. This metal has a direct effect on the gut mucosa, causing early pathologic findings. Hemorrhagic necrosis of the proximal gastrointestinal tract is a common finding at autopsy. Changes in the liver range from no-change to swelling, hemorrhagic periportal necrosis, and fatty degeneration. Peripheral portions of the liver are affected to a greater extent than the central hepatic cells, because of their proximity to the portal vein. The myocardium also shows evidence of fatty degeneration following excess iron ingestion (Eisen *et al.*, 1990).

Lead poisoning is thought to be due to its ability to combine with sulfhydral groups on proteins. The primary organs of attack are the brain and the peripheral nervous system, bone marrow, kidney and liver. Renal tubular function is altered, resulting in a variety of renal-related disorders (Garrettson, 1990).

Chromium poisoning from chromium trioxide or chromium salts, if ingested, is characterised by, amongst others, renal failure due to toxic nephritis.

Abnormal doses of copper results in deposition of copper in the liver, brain and kidneys, resulting in renal tubular abnormalities and chronic active hepatitis.

Ingested Zinc, and especially zinc chlorides, leads to erosive pharyngitis and esophagitis (Hall and Robertson, 1990).

In general, if the heavy metal compound is highly ionisable, the typical corrosive gastrointestinal symptoms result. Subsequent advancement is evidenced by hepatic, renal or other system impairment (Hall & Robertson, 1990).

Sodium chloride, although an essential and ubiquitous salt, has also been demonstrated to have toxic effects when ingested in abnormally high doses. Sodium, an essential mineral, is rapidly absorbed throughout the gastrointestinal tract. The kidney is the primary organ for regulation of sodium output. The renal threshold for sodium is 110-130 $mEq.L^{-1}$. Chloride, the other component of salt, is essential for water balance, acid-base balance and serum osmolality. Salt poisoning may cause acute renal tubular necrosis. The toxic oral dose of salt is 0.5-1.0 $g.kg^{-1}$, with the fatal amount being in the range 1-3 $g.kg^{-1}$ (Mofenson & Caraccio, 1990).

6.1.4 *Artemia salina* bioassay

Gross feeding studies employing a variety of test animals, and consequent pathological evaluations should be one of the final steps in the evaluation of a potential toxin. There exists a simple bioassay utilising the brine shrimp, *Artemia salina* being one of a range of simple, rapid and inexpensive bioassays presently used for the pharmacological testing of active constituents. *A. salina* has been used previously in the analysis of pesticide residues, mycotoxins, stream pollutants, anaesthetics, dinoflagellate toxins, morphine-like compounds, toxicity of oil dispersants, cocarcinogenicity of phorbol esters and toxicants in marine environments (Meyer *et al.*, 1982).

6.2 RESEARCH OBJECTIVES

The above review documents available literature on the preliminary safety testing of selected toxic compounds in SCP sources. As can be imagined, the list of possible toxicologic tests is exhaustive, both in terms of listing and practical input. Each SCP source has its peculiar target areas of toxicological constraints, these areas depending on a variety of factors, including medium composition and source, prevailing physical and chemical conditions, product processing and destination. This phase of research was consequently undertaken to establish the toxicological constraints of tannery-effluent grown *Spirulina* destined for animal consumption. The following areas were of primary concern:

1. Is the nucleic-acid content within the safety limit?

2. Does *Spirulina* accumulate pesticides due to run-off from the surrounding agricultural land?

3. Are minerals and heavy metals accumulated from the ponded tannery effluent?

4. Are there active toxic constituents in the biomass detectable in a bioassay with *Artemia salina*?

5. Can the dried biomass be used as an adequate feed-supplement in the rearing of chickens, and are there any manifestations of toxicity in the gross chemical and histopathology of target organs?

6.3 MATERIALS AND METHODS

6.3.1 Nucleic Acid Content

The nucleic acid concentrations were determined according to the method of Plummer (1978).

6.3.1.1 DNA by the Diphenylamine Reaction

A 10 mg/ml suspension of dried *Spirulina* biomass was prepared in buffered saline (0.15 mol.L^{-1} NaCl, 0.015 mol.L^{-1} Sodium Citrate, pH 7.0). The suspension was sonicated for 1 min. (4x15 sec pulses), and microfuged to clarify the resulting solution. A ten-, hundred-and thousandfold dilution was prepared from the test stock solution.

A 0.2 mg.ml^{-1} standard stock solution was prepared using Herring Sperm DNA in buffered saline solution. Dilutions ranging from 0.2-0.0 mg.ml^{-1} were prepared using saline solution.

2 ml of the test solutions were pipetted into test tubes. 4 ml Diphenylamine solution (1 g diphenylamine in 100 ml glacial acetic acid + 2.5 ml H_2SO_4 [v/v]) was added and rapidly stirred. The test tubes were heated on a boiling water bath (10 mins) and then cooled to room temperature. The absorbance at 595 nm was read. A standard curve was constructed from the absorbance values of the standard solutions, and the DNA concentration of the test solution was extrapolated from the curve.

6.3.1.2 RNA by the Orcinol Reaction

A suspension of dried biomass was made as described above, and a dilution series was prepared.

A 0.2 mg.ml^{-1} standard stock solution of RNA was prepared as above. A dilution range was prepared.

The orcinol reagent was prepared by dissolving 0.1% $FeCl_3.6H_2O$ in concentrated HCl (w/v). 3.5 ml orcinol in ethanol (6%w/v) was added to 100 ml of this solution, and stirred.

2 ml Test solutions were pippetted into test tubes. 3 ml Orcinol reagent was added, and the resulting solution was heated on a boiling water bath (20 mins). The solutions were cooled to room temperature, and the absorbance read at 665 nm. A standard curve was constructed, and the RNA concentration of the test solution was extrapolated from the curve.

6.3.2 Pesticide Accumulation

A sample of dried and milled *Spirulina* biomass was sent in a frozen state to the South African Bureau of Standards (SABS) in Pretoria, South Africa, for evaluation of the pesticide content by gas liquid chromatography.

A range of pesticides was tested for, spanning the three groups of pesticides commonly in use today, viz organochlorides, organophosphates and synthetic pyrethroids. A specific test for DDT and its isomers was also performed.

Recovery determinations were undertaken by adding known amounts of different pesticides to portions of the sample and analyzing these concurrently with the sample.

6.3.3 Mineral & Heavy Metal Content

Duplicate masses of 2 g *Spirulina* biomass (corresponding to 1.546 g dried weight) were placed in 100 ml volumetric flasks with a few glass beads. 10 ml nitric acid was added, followed by the addition of 30 mixed perchloric-sulphuric acid (3:1). Digestion was continued over heat until the thick, white acid-fumes lifted from the pale yellow remaining liquid. This remaining solution was cooled to room temperature, followed by the addition of 50 ml d H_2O. The solution volume was reduced to 25 ml by boiling, and then made up to 50 ml with d H_2O.

Heavy metal content was then determined by atomic absorption. Cr, Fe, Cu, Mg, Zn and Na contents were quantified using an air/acetylene gas mixture (Temperature = 2500°C). Ca and Al content were determined using a nitrous oxide/acetylene gas mixture (Temp = 3000°C). NaCl concentration was determined using a standard refractive index/salinometer.

6.3.4 Brine Shrimp Bioassay

A method modified from Meyer *et al.* (1982) was used in the bioassay of active constituents of the *Spirulina* biomass. Autoclaved sea-water (35 ppm) was used as

hatching and incubation media. *Artemia salina* was obtained in dried egg form. The brine shrimp eggs were hatched in a two compartment flat container, one compartment covered with foil (the dark compartment), and the other being left uncovered (the light compartment). A plastic divider perforated with 1 mm bore holes was used to separate the two compartments. The eggs were placed in the dark container, within which they hatch into nauplii larvae. The larvae, being positively phototactic, swim from the dark compartment, through the bored divider, into the light compartment. These larvae can then be used in the biological toxicity experiments.

The first experiment involved the toxicological testing of the water-soluble components in the *Spirulina* biomass. Two stock solutions of dried *Spirulina* were prepared, with concentrations of 0.5 and 1 $mg.ml^{-1}$ biomass in autoclaved sea-water. Triplicate stock solutions were made, and dilutions of 0-0.5 $mg.ml^{-1}$ (for 0.5 $mg.ml^{-1}$) and 0-1 $mg.ml^{-1}$ (for 1 $mg.ml^{-1}$) were prepared in a total volume of 5 ml. Autoclaved sea-water was used as the diluent. Ten nauplii larvae were used per experimental tube. Each biomass concentration tested was performed in five replicate tubes. Inoculated larvae in the various dilutions of *Spirulina* were incubated at 25°C. The number of moving larvae were counted after 12 and 24 hrs by siphoning the larvae into a pasteur pipette, and counting them against a white-light source.

The second experiment involved the testing of the cell-free extract of the *Spirulina* biomass. Stock solutions of 10 $mg.ml^{-1}$ *Spirulina* in autoclaved sea-water were prepared in triplicate. Cell-free extracts were prepared by sonicating the solutions for 4 x 13 second pulses, followed by centrifugation (6000 rpm/10 mins). These solutions were diluted to 1 $mg.ml^{-1}$ biomass concentration, and various dilutions of the 1 $mg.ml^{-1}$ solution were prepared. Five replicate tubes were used for each test concentration. Ten nauplii larvae were inoculated into these dilutions, and incubated at 25°C. The numbers of larvae moving were counted at 12 and 24 hrs.

6.3.5 Chicken Feeding Trial

6.3.5.1 Feeding Trial with *Spirulina*

Sixty newborn cockerels were obtained from Tokai Breeders in Cape Town, South Africa. The chicks were fed commercial chicken rearing mash for 7 days prior to the start of the feeding trial with *Spirulina*. After 7 days, they were divided into three groups of 20 chickens each. Group 1 was used as a control, being fed commercial grower's mash. Groups 2 and 3 were fed commercial grower's mash supplemented with 10 and 50% *Spirulina*, respectively. Groups 1 and 2 were fed their respective rations continually for 21 days. The chickens fed the 50% *Spirulina* supplement, however, suffered from mild diarrhoea, presumably due to the high NaCl concentration in the milled *Spirulina* feed. The diarrhoea was stopped by staggering the feeding regime with unsupplemented commercial rations every 5 days for 5-day periods.

The feed consumption and weight gain was monitored throughout the duration of the feeding trial. The Feed Conversion Ratio (FCR) could thus be determined. The FCR is a ratio of the feed consumed/unit weight gained. The greater the FCR, the less efficient the feed source.

The skin and feather pigmentation was also noted.

After 21 days the chicks were killed by injection with 1 ml Euthanase^{R}. All chicks died within 5 minutes after administration of the drug. Animals were immediately dissected and examined for gross morphological abnormalities.

6.3.5.2 Organ Dry Weights

The entire heart, liver, kidney and spleen of 5 chicks from each feeding group were removed. These were weighed (wet weight), dried at 60°C for 48 hrs, dehydrated in a desiccator for 3-5 hrs, and then reweighed. The dry weights of the organs from the different feeding groups could thus be determined, as any induced abnormalities present may be reflected in differences in the organ weights.

6.3.5.3 Liver Mineral & Heavy Metal Content Determination

Dried liver samples were analyzed for heavy metal content by atomic absorption (AA).

The dried liver samples were prepared for AA by acid digestion according to the above protocol. Five tissue samples from each of the feeding groups were analyzed for Cr, Fe, Cu, Mg, Zn, Ca, Al and Na.

6.3.5.4 Histological Examination

Sections of the heart, liver, kidney, spleen, and small intestine of 5 chicks from each of the 3 feeding groups were fixed in 10% formaldehyde in phosphate-buffered saline solution. Tissue sections were allowed to fix for 3 days, and were then passed through a series of alcohol and chloroform dehydrations, followed by final embedding in paraplast. The embedded tissue sections were then sent to a specialist veterinary pathology laboratory in Cape Town. Samples were sectioned and stained with haematoxylin-eosin stain solution. The completed slides were examined for histopathological differences between the chickens from the different feeding groups.

6.3.5.5 Statistical Analysis

One-way analysis of variance (ANOVA) determinations were performed the Statsgraphics statistical software programme, in order to determine whether the variations in the test animals were independent of the effluent-grown *Spirulina* supplementation. A p-value of < 0.05 indicates rejection of this null hypothesis at a 95% confidence level and 5% level of significance.

6.4 RESULTS

6.4.1 Nucleic Acid Content

The DNA standard concentrations plotted against absorbance at 575 nm yielded a linear regression curve with $x = y/0.77$. Substitution of absorbance values in this formula yielded a mean DNA concentration of 3.7 g DNA.kg^{-1} dry weight, which corresponds to 0.4% DNA in the *Spirulina* biomass.

The RNA standard concentrations plotted against absorbance at 665 nm yielded a linear regression curve with $x = y/8.72$. Substitution of absorbance values in this formula yielded a mean RNA concentration of 37.8 g RNA.kg^{-1} dry weight, which corresponds to 3.8% RNA in the *Spirulina* biomass.

Total nucleic acids therefore make up 4.2% of the total dry weight of the harvested *Spirulina* biomass.

6.4.2 Pesticide Content

The gas-liquid chromatography test-report from the SABS (Test Report No. 311/88151/H346) states that none of the more commonly encountered pesticides could be detected down to a level of 0.05 mg.kg^{-1}. Pesticides tested for included Aldrin, Dieldrin, Endrin (organochlorides), Malathion, Parathion, Diazinon, Chlorpyrephos, Pyrenephos (organophosphates), Permethrin, Cypermethrin, alpha-Methrin, and Fenvelarate (synthetic pyrethroids). Unidentified substances were, however, found to be present.

DDT and its isomers could not be detected down to a level of 0.02 mg.kg^{-1}.

Recovery determinations (by spiking with known concentrations of pesticides) yielded the following results, shown in Table 6.2.

Table 6.2 Recovery determinations of the three main pesticide groups.

Pesticide group	Level ($mg.kg^{-1}$)	Recovery (%)
Organophosphates	0.1	80
Organochlorides	0.1	102
Synthetic Pyrethroids	0.1	88

The recovery information shows that some degradation of the pesticides does occur during sample preparation.

6.4.3 Mineral & Heavy Metal Content

The mineral and heavy metal content of the dried and milled *Spirulina* biomass as determined by atomic absorption is shown in Table 6.3. 1.546 g Dried biomass was used in the digestions. Results are averages of duplicate AA runs.

Table 6.3 Mineral and heavy metal content of the milled *Spirulina* biomass.

Metal	ppm in Digest	$ppm.g^{-1}$ Dry Weight	mg $Metal.kg^{-1}$ Dry Weight
Cr	0.3	0.2	20
Fe	7.8	5.0	500
Cu	0.05	0.03	3
Mg	90.0	58.2	5820
Zn	2.7	1.8	180
Ca	21.5	13.9	1390
Al	7.8	5.0	500
Na	1640	106	10 600
NaCl	120	78	7 800

6.4.4 Bioassay with Artemia salina

The results from the 2 quadruplet runs utilising 0.5 and 1.0 mg.ml^{-1} maximum biomass concentrations were combined and results were averaged, yielding a sample sizes of 10 (0-0.5 mg.ml^{-1}) and 5 (0.6-1.0 mg.ml^{-1}). The water-soluble biomass had no observable toxic effects, with a >90% survival of the larvae obtained after 24 hrs incubation, at all biomass concentrations tested (Figure 6.1.).

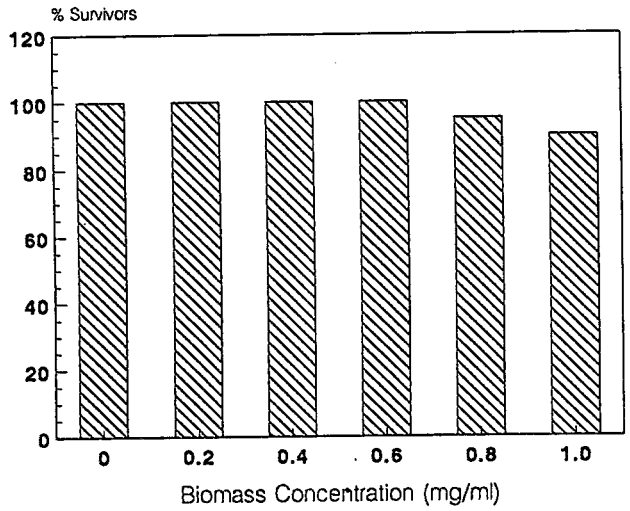

Figure 6.1 Survival of *A.salina* nauplii larvae at 24 hrs, after exposure to varying concentrations of *Spirulina* biomass.

The sonicated biomass, as expected yielded a toxic test solution (Figure 6.2), by virtue of the elevated concentration of cellular components.

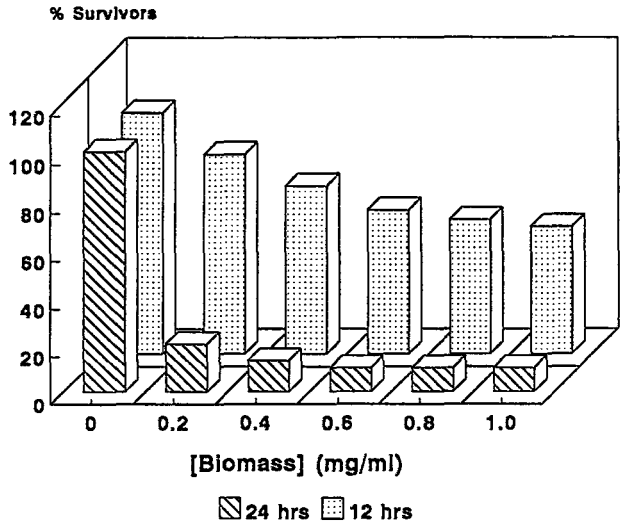

Figure 6.2 Survival of *A.salina* nauplii larvae at 12 and 24 hrs, after exposure to varying concentrations of lysed *Spirulina* biomass.

Statistical analysis reinforced the above observations, and results of the anova tests are shown in Table 6.4.

Table 6.4 Analysis of variance induced by dosing of *A.salina* growth medium with effluent-grown *Spirulina*.

Sample	Degrees of freedom	F-ratio	p-value
Water soluble	5	2.200	0.1222
Lysed (12hrs)	5	29.367	0.0000
Lysed (24hrs)	5	9.515	0.0007

6.4.5 Chicken Feeding Trial

6.4.5.1 Feeding & Weight Gain

The feed consumption of the various feeding groups did not exhibit noticeable differences (Figure 6.3).

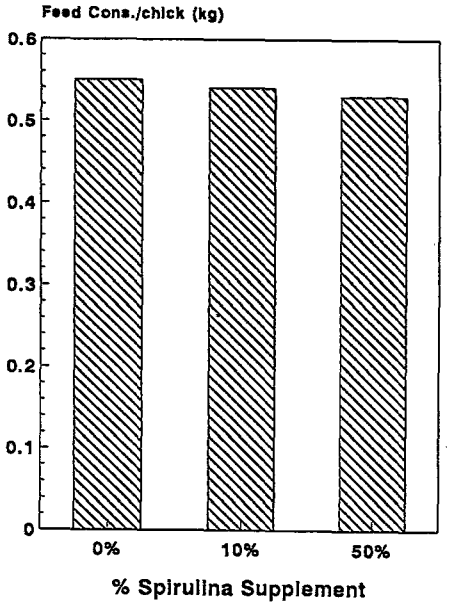

Figure 6.3 Feed Consumption of the three feeding groups of chickens.

The different feeding groups also exhibited little variation in their growth rates over the 21 day period (Figure 6.4).

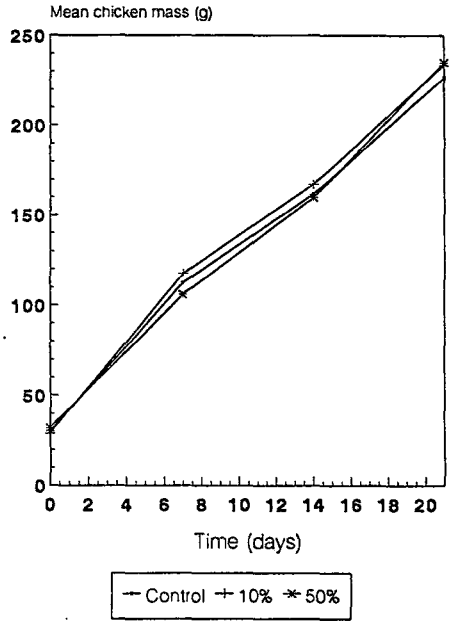

Figure 6.4 Growth rates of the three feeding groups of chickens.

The FCR for the different feeding groups are shown in Figure 6.5. Again, as with the feed consumption and growth rates, little variation is exhibited.

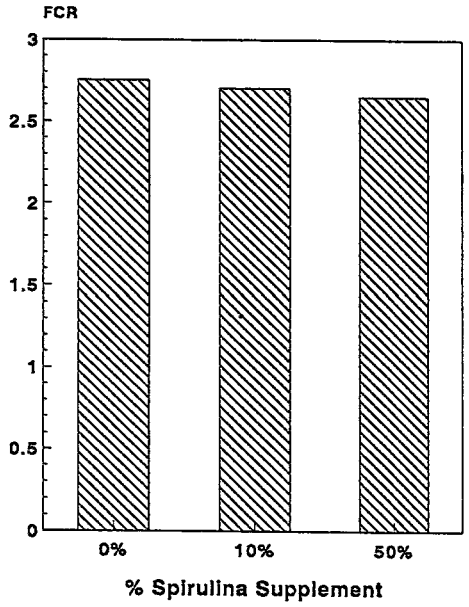

Figure 6.5 Feed Conversion Ratios (FCR) for the three different feeding groups.

6.4.5.2 Organ Dry Weights

The wet weights of the hearts, livers, kidneys and spleen from the different feeding groups are shown in Figure 6.6. The corresponding dry weights are shown in Figure 6.7. Both sets of data show the same trends.

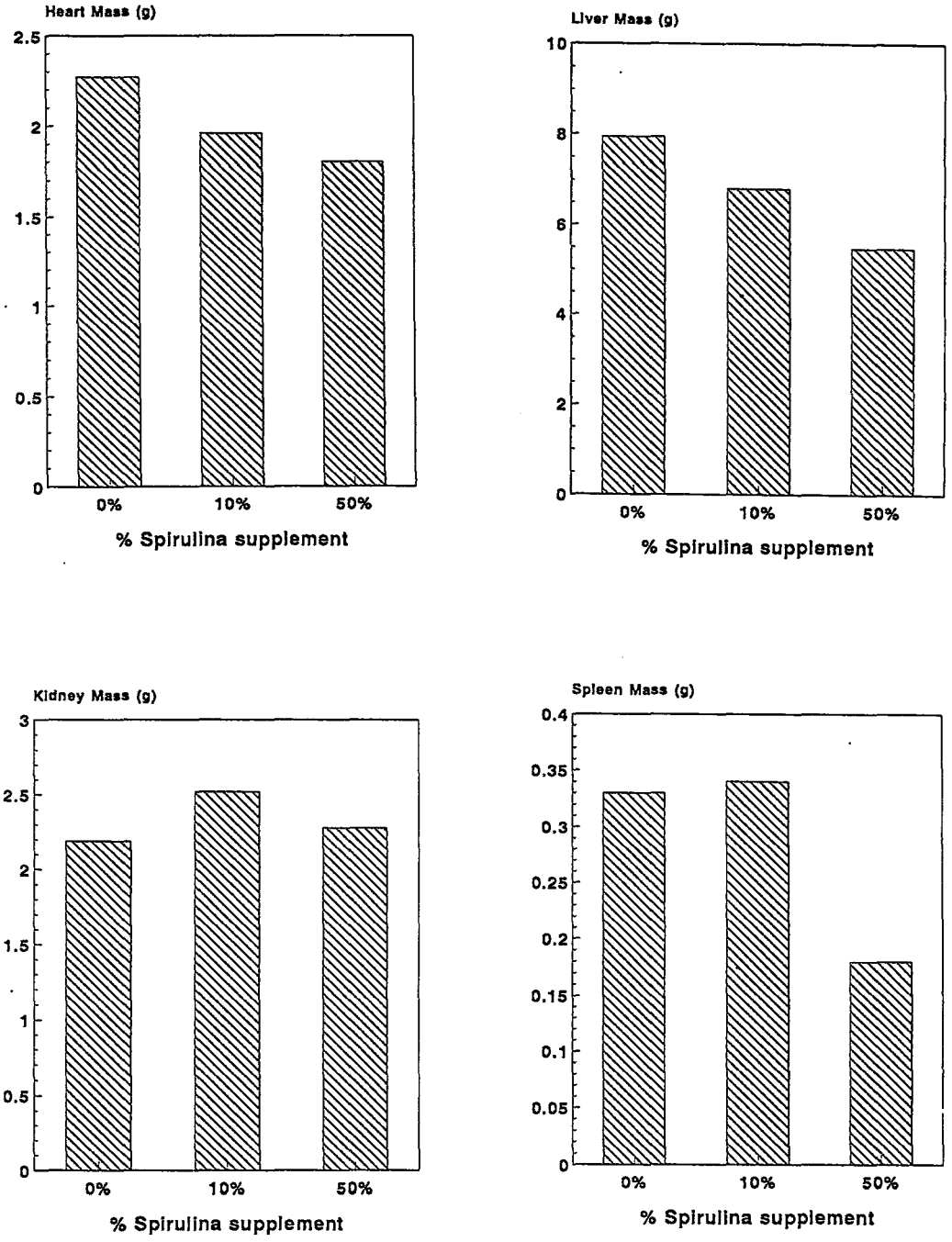

Figure 6.6 Wet weight analysis of the hearts, livers, kidneys and spleens from the three chicken feeding groups.

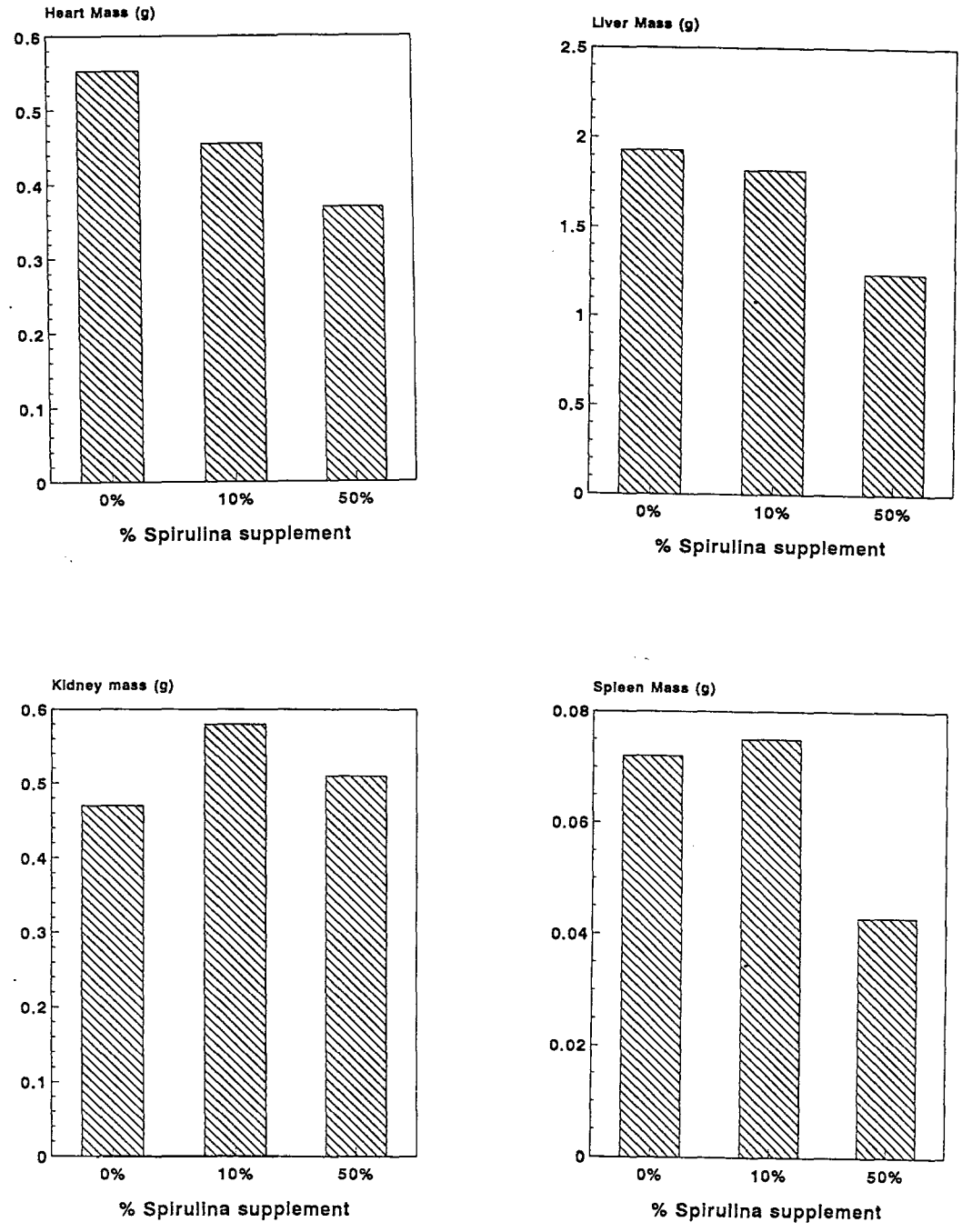

Figure 6.7 Dry weight analysis of the hearts, livers, kidneys and spleens from the three chicken feeding groups.

Statistical analyses of the results presented in Figures 6.6 and 6.7 are shown in Table 6.5.

Table 6.5 Analysis of variance between the organ wet- and dry weights of chicken fed a range of *Spirulina*-supplemented test diets.

Analysis	Test Organ	Degrees of Freedom	F-ratio	p-value
Wet weight	Heart	2	3.570	0.608
	Liver	2	7.834	0.0067
	Kidney	2	0.777	0.4618
	Spleen	2	4.055	0.0451
Dry weight	Heart	2	13.426	0.0009
	Liver	2	18.711	0.0002
	Kidney	2	2.031	0.1739
	Spleen	2	2.509	0.1229

With the exception of the kidneys, there is a general trend of a decrease in organ wet and dry weight with an increase in *Spirulina* supplement in the diets. Anova tests show that only the liver exhibited the most unequivocably significant decrease in both wet and dry weights with an increase in supplementation with *Spirulina*.

6.4.5.3 Liver Metal Accumulation

The liver shows the most significant decrease in weight. This, coupled with the fact that the liver is one of the main target organ for toxic compounds and metals, led to the dried liver samples being subjected to acid digestion for the determination of the mineral and heavy metal content by atomic absorption. The results are presented in Figure 6.8.

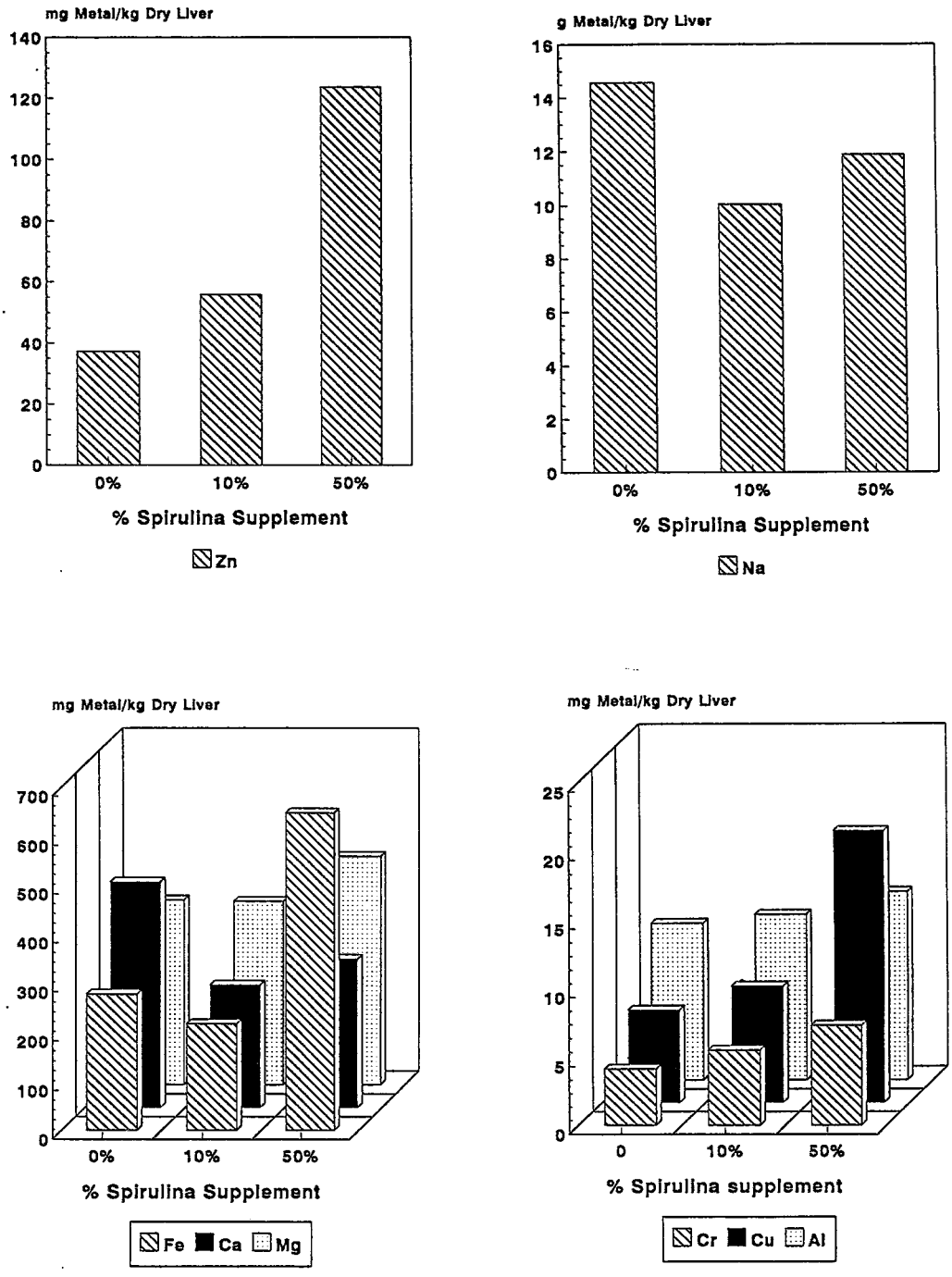

Figure 6.8 Mineral and metal content of the dried liver samples as determined by atomic absorption spectroscopy.

Figure 6.8 shows the mineral and heavy metal content in a standardized format (in mg metal.kg^{-1} dry weight). The metal content of the individual livers (each an average of five), however, are shown in Table 6.6.

Table 6.6 Mineral and heavy metal content (ppm) in acid digests of the livers of chickens fed *Spirulina*.

Metal	Control	10% Supplement	50% Supplement
Cr	0.16	0.20	0.18
Cu	0.26	0.31	0.49
Al	0.44	0.44	0.34
Zn	1.44	2.04	3.06
Fe	10.7	7.92	16.0
Mg	14.5	13.6	11.5
Ca	17.7	9.1	7.48
Na	562	366	294

Statistical analyses of the mineral and metal content in the liver digests are shown in Table 6.7.

Table 6.7 Analysis of variance between the liver mineral and metal contents of chickens fed a range of test diets.

Mineral/Metal	Degrees of Freedom	F-ratio	p-value
Cr	2	1.200	0.3349
Fe	2	15.975	0.0004
Cu	2	10.840	0.0020
Mg	2	4.087	0.0443
Zn	2	45.945	0.0000
Ca	2	10.436	0.0024
Al	2	1.020	0.3897
Na	2	8.309	0.0054

There is a concommitant relationship between increasing concentrations of *Spirulina* supplementation and Fe, Cu, Zn and Ca contents in the livers of the test animals.

6.4.5.4 Pathological Examination

Typical examples of the histopathology of sections of the hearts (Figure 6.9), livers (Figure 6.10), kidneys (Figure 6.11), spleens (Figure 6.12) and intestines (Figure 6.13) fromthe three feeding groups are shown in the respective figures. Annotations and descriptions are given with each figure. Photographs labelled **a**, **b**, and **c** refer to the control, 10% and 50% feeding groups, respectively.

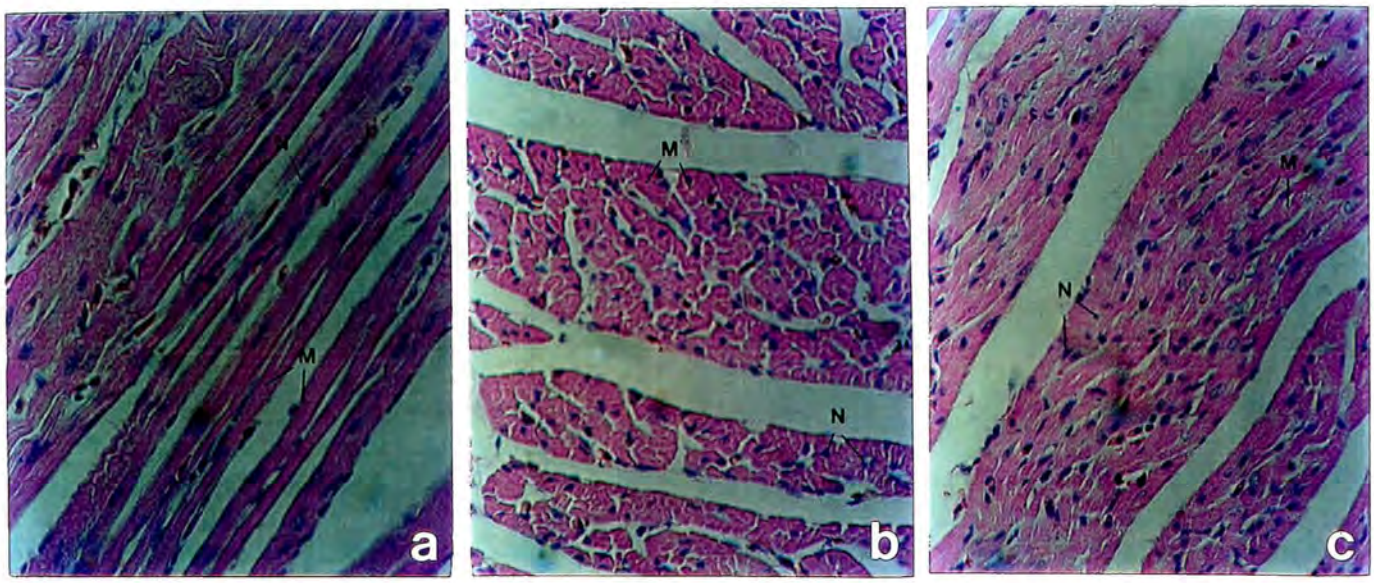

Figure 6.9 Sections of the hearts from the three chicken feeding groups (mag. = 100X). The myocardial fibres in all the examined samples spanning the three feeding groups appear to be faintly granular, but the change is mild and does not appear to be significant. No other significant lesions are noticeable in any of the specimens. (**M**yofibrils; **N**ucleus)

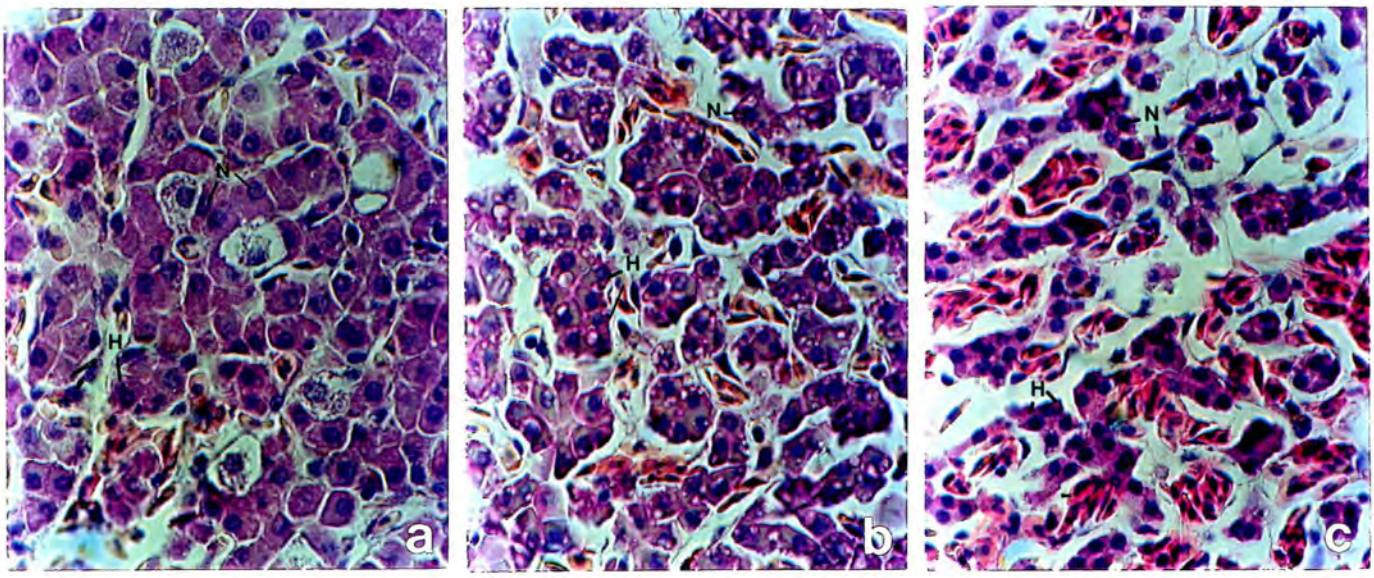

Figure 6.10 Sections of the livers from the three chicken feeding groups (mag = 160X). There are no significant morphological lesions in the control and 10% feeding groups. One specimen from the 50% feeding group, however, exhibited atrophy of the hepatocyte chords, but this condition was not noted in any of the other specimens examined. Another specimen was shown to contain a few urate crystals in the lumen of some of the cortical tubes. This may be due to dehydration caused by the high concentration of NaCl in the biomass. Again, this condition was not noted in any of the other examined specimens. (**N**uclei; **H**epatocytes)

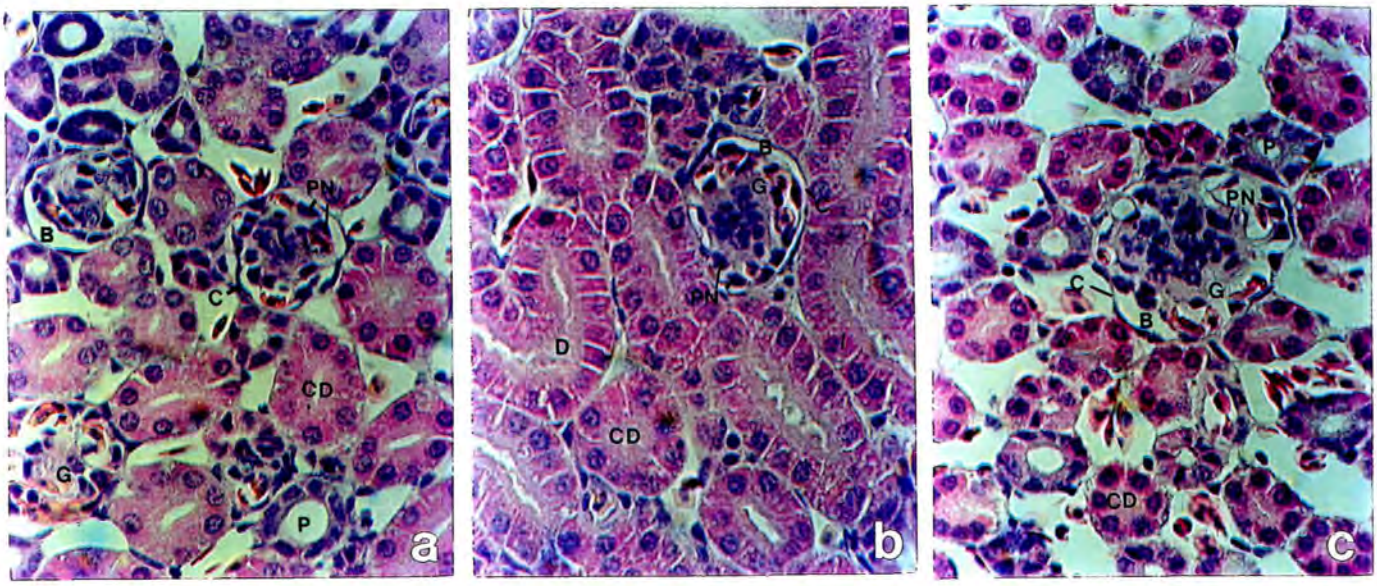

Figure 6.11 Sections of the kidneys from the three chicken feeding groups (mag. = 160X). Generally, there are no significant lesions in any of the feeding groups. Some of the individual renal tubular epithelial cells in the 50% feeding group exhibit slight degeneration. This, however, is probably due to mild dehydration caused by the high NaCl concentration in the *Spirulina* biomass. (**B**owman's capsule; **C**apsule epithelium; **C**ollecting **D**uct; **D**istal convoluted tubule; **G**lomerulus; **P**roximal convoluted tubule; **P**odocyte **N**uclei)

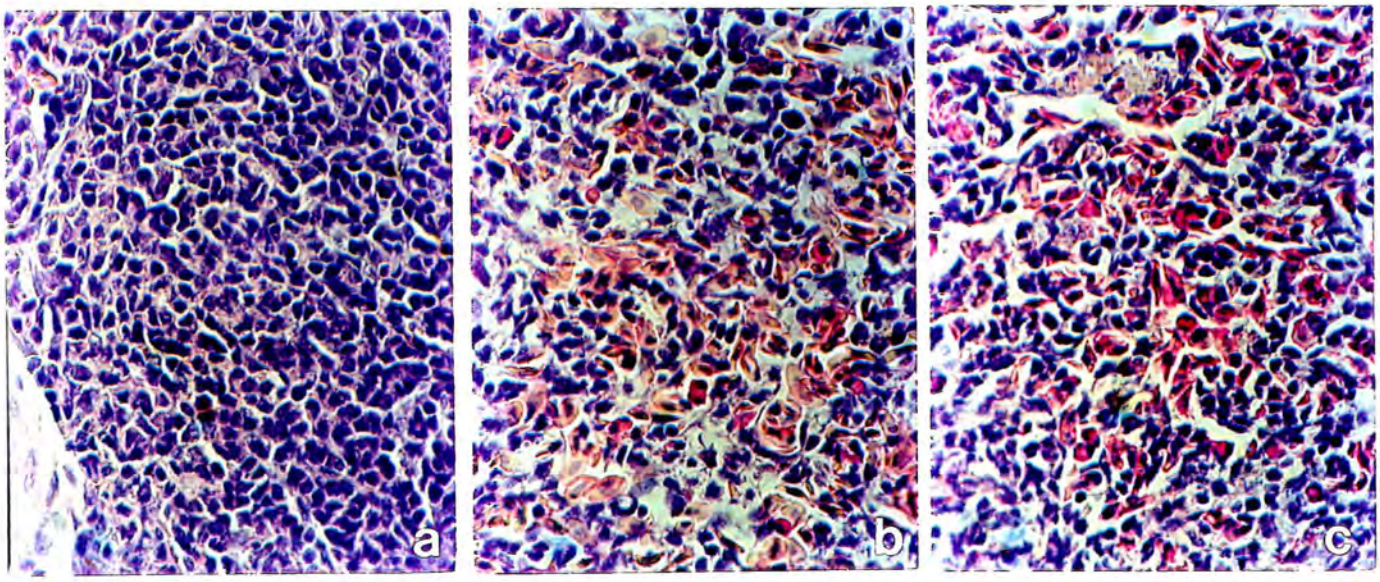

Figure 6.12 Sections of the spleens from the three chicken feeding groups (mag. = 160X). A few of the specimens from the control and 10% feeding groups exhibit mild lymphoid hyperplasia, and only the control group exhibits an increase in the number of eosinophils. The spleens of the 50% feeding group, on the other hand, show no significant lesions. The density of the white and red pulp appears unaltered in the different experimental groups.

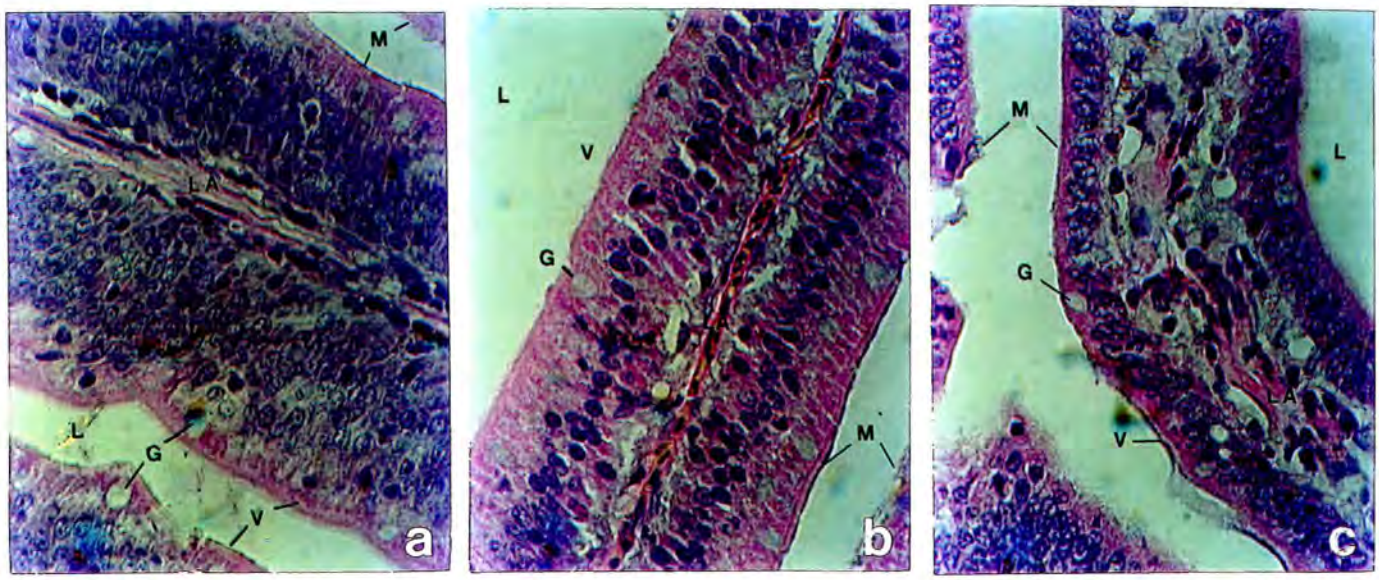

Figure 6.13 Sections of the intestines from the three chicken feeding groups (mag. = 160X). No significant lesions were noted in any of the specimens examined. (**G**oblet cell; **L**umen of intestine; **LA**cteal; **M**ucous membrane; **V**illus)

6.5 DISCUSSION

Methods for assessing the toxicity of a novel protein source have been developed over the years, combining a variety of biochemical, physiological, haematological, immunological, morphological and histopathological techniques, in a range of test animals (Becker, 1980).

With the appearance of SCP sources, the principles and techniques of classic toxicology (which dealt with the evaluation of quantitatively minor ingredients) were combined with the practical limitations imposed by the nature and intended use of the material under test. The numbers of toxicological tests available are exhaustive, and it is thus not advisable to embark on a full scale, definitive programme until some preliminary screening is done (van der Wal, 1980). The nutritional analyses presented in the previous chapter presents the first evaluation: that of nutritional feasibility for the target animals under study. In order to give a preliminary go-ahead for animal studies, an initial survey of the prime toxicological constraints on the use of effluent-grown *Spirulina* as a protein supplement was needed.

6.5.1 Nucleic Acid Content

The first evaluation, that of nucleic acid content, is an inherent toxicological constraint of SCP, including species grown in defined, sterilized media. The dried and milled *Spirulina* was found to contain 0.4% DNA and 3.8% RNA, with a total nucleic acid content of 4.2% (dry weight). This is in the range found by Durand-Chastel (1980), where *Spirulina* from Lake Texcoco (Mexico) contained 0.7-1.0% DNA, and 2.2-3.5% RNA. Nucleic acid content of microalgae are, however, in the lower range of other SCP sources (Litchfield, 1979), and this lightens the toxicological load.

6.5.2 Pesticide Content

The pesticide content of Spirulina was also of toxicological concern, as pesticides may have accumulated due to run-off from the surrounding agriculturally intensive land. A sample of biomass was prepared for chemical pesticide analysis by the Pesticide Residue Laboratory of the SABS in Pretoria. None of the organochlorides, organophosphates and synthetic pyrethroids they probed for were detected. The test for DDT and its isomers was also negative. The recovery information (Table 6.2) does, however, indicate that some loss of residue may occur during sample preparation. A loss of 20% was obtained with the organophosphate compounds. A loss of 12% was obtained with the synthetic pyrethroids. The organochloride pesticides (DDT and its isomers, *inter alia*), however, exhibited the greatest recovery (102%). These organochloride pesticides are the most persistent and stable in the environment, so the zero detection, coupled with the 102% recovery, indicates that there are no detectable amounts (to a level of 0.02 $mg.kg^{-1}$) present in the *Spirulina* biomass.

The toxicological constraint posed by the potential pesticide accumulation can now be waved aside.

6.5.3 Mineral & Heavy Metal Content

Due to problems encountered with the standard spectra, those metals listed in Table 6.3 were the only ones tested for. Nevertheless, there seems to be an indication of some heavy metal accumulation by the *Spirulina* biomass (Table 6.3). The toxic effects of ingestion of these amounts of heavy metals can only be undertaken in feeding trials, and consequently the heavy metal content of the biomass will be discussed in context of the heavy metal accumulation by the target organs of chickens.

6.5.4 *Artemia salina* Bioassay

The survival rate of the brine shrimp larvae (Figure 6.1) indicates that the *Spirulina* biomass does not contain active amounts of soluble biotoxic compounds. The brine shrimp larvae exhibited a >90% survival at all concentrations tested. As expected however, the cellular components of *Spirulina* were toxic to the brine shrimp larvae (Figure 6.2).

This low biogenic activity exhibited by the water-soluble (non-cellular) components *Spirulina* biomass is reinforced by the overwhelming lack of information on toxins produced by this species of cyanobacterium. Although there is an abundance of literature on the toxic compounds produced by cyanobacteria (Pohland *et al.*, 1990), none of the authors mention toxins produced by *Spirulina*. This, coupled with the observed low biotoxicity exhibited by the *Spirulina* biomass, leads us to conclude that the WTC source of *Spirulina* has no active biotoxic compounds.

The above analyses and information presented a preliminary indication of safety of the tannery-effluent generated *Spirulina* biomass. This initiates the go-ahead for the toxicological evaluation of the biomass in test animals.

6.5.5 Chicken Feeding Trial

The chicken was chosen because of its universal use as a target animal for toxicological assessment, and because of its relatively fast growth rate (ensuring early manifestation of toxicological symptoms).

Over the three week feeding trial the feed consumptions of the three feeding groups are similar (Figure 6.3). The typical symptom of malnutrition, that of a higher feed consumption, is thus not present. The rates of weight gain of the three groups are nearly identical (Figure 6.4), as are the Feed Conversion Ratios (Figure 6.5). It can thus be concluded that increasing supplements of *Spirulina* does not alter the amount of available protein, and thus places no nutritional constraints on the use of *Spirulina* as a protein supplement in chickens. Other authors have also shown that effluent-grown microalgae are an acceptable source of protein for chickens. Shelef *et al.*, (1980) reports that extensive nutritional experiments with broiler chicks and laying hens showed that when High Rate Algal Pond (HRAP) flocculated drum- dried algae replaced 25% of soybean meal in the diet of poultry, the rate of weight gain and the general health of the birds were comparable to those obtained with commercial feed. Mokady *et al.*, (1980) used wastewater-grown *Oocystis*, *Scenedesmus*, *Micractinium* and *Chlorella-Euglena* biomass in feeding trials with chickens. They found that all the algal species tested could successfully replace 25% of the soy protein. In some cases, a 50% algal supplement also proved nutritionally adequate. Similar results were found earlier by other authors (e.g. Combs, 1952; Grau & Klein, 1957; Yannai *et al.*, 1980). The general consensus seems to be that an algal supplement of up to 10% in chicken diets is not deleterious, and may bestow, in some cases, a nutritional advantage to the chicken rations.

The chickens fed with the *Spirulina* supplemented rations show a marked increase in overall body pigmentation with increasing levels of algae in the diet, with the beak, feet and feathers developing a yellow tint. This is in agreement with that found by other authors (Lipstein & Hurwitz, 1980, Grau & Klein, 1957). *Spirulina* can thus be used as a pigment source in chickens, geared towards markets where this skin colouration is advantageous.

During the feeding trial period, the only abnormality noticed was that the 50% feeding group exhibited slight diarrhoea and an abnormally high water consumption. This (later confirmed by a histopathological examination) was due to the high NaCl concentration of the biomass. The diarrhoea was arrested by the staggering of the 50% diet with an unsupplemented ration.

After three weeks, the chickens were killed and their organs removed for weight analysis. As shown in Figures 6.6 & 6.7, the general trend is that of a decrease in both wet and dry organ weights with an increase in the concentration of supplemented *Spirulina*. This effect was also observed by Becker (1980) in a feeding trial with *Scenedesmus*-fed rats, although the effects were less pronounced than those observed in the feeding trial reported here.

The livers from the 50% feeding group exhibited the greatest weight-reduction response of all the organs examined. This, coupled with the fact that the liver is one of the main target organs for toxic compounds, prompted the determination of the heavy metal content of the dried liver samples.

From Figure 6.8 it seems that Cr, Cu, Al, Zn, Fe and Mg levels in the liver increase with an increase in *Spirulina* supplement. These units, however, are given as grammes of metal per kg liver (dry weight). The raw data (Table 6.6) indicate that, quantitatively, there are little differences in total heavy metal content of the livers from the various groups. Anova tests, however, indicate signifgicant accumulation of Fe, Cu, Zn, and Ca. Due to the already low concentration of copper in the biomass (3 $mg.kg^{-1}$ dry weight), and the known low toxicity, even beneficial properties of zinc (Duncan, pers. comm), the accumulation of these two metals do not seem to pose a major threat. The effects of accumulation of these elements, however, can only be elucidated in a histopathological study and will be discussed later.

Direct examination of the toxic effects of compounds can be done in two ways : Firstly, assay for the compounds in question. This is a tedious task, notwithstanding the number of tests and practical input needed to cover such a broad field. Selectivity is thus of major practical and economic importance. The second method employs an indirect approach: evaluation of

the effects of potential toxins in target areas. Target organs, in this case, include the heart, spleen and intestine, and, of considerable more toxicological bearing, the liver and kidney. These are broad-range target organs for a variety of toxins by virtue of their position, structure, biochemistry and cellular functions. Thus any toxicological effect should be manifested in the histopathology.

The prepared samples were examined for any abnormalities. The heart tissue from all three feeding groups were normal (Figure 6.9). The striated muscle, and the numbers and distribution of the nuclei appear normal. The myocardial fibres in all the chickens appear faintly granular, but the change is mild and does not appear to be significant.

The livers from all the feeding groups appear to be normal (Figure 6.10). This was our main area of concern in the histopathological study, as the liver is more succeptable to injury because, as a portal of entry to the systemic circulation and the other tissues, it is exposed to higher concentrations of toxic substances prior to their dilution in the systemic circulation. Once within the liver, foreign compounds readily come into intimate contact with liver cells, facilitated by the sinusoidal structure of this organ. The large and diverse metabolic capabilities of the liver enable it to metabolise a variety of compounds, which may result in toxic injury. The liver also plays an extensive role in intermediary metabolism, and interference of this by foreign compounds may also make the liver a target organ (Timbrell, 1988).

The liver sections from all the feeding groups appear normal. The parenchyma cells seem unaffected. The cell volumes are maintained in all the examined samples, which indicates unrestricted blood flow. Swollen liver cells is one of the early signs of liver damage, indicating blocking of the sinusoids, leading to restricted blood flow through the liver. The lobules also appear of normal shape and spatial arrangement. The cells around the central vein, and especially the area where several acini meet, are the most succeptable to damage (Timbrell, 1988), and these, too, appear unaffected.

The final pathology report noted that the hepatocytes in all the chickens were slightly atrophic. This is, however, probably due to some deficiency in the basic commercial ration,

as this abnormality appeared to the same degree in all the liver samples, including those of the control animals fed only the commercial ration.

The kidney is another prime target organ for toxic compounds. There seem to be very few morphological differences in the kidneys from the different feeding groups (Figure 6.11). It was noted that some of the individual renal tubular epithelial cells in the 50% feeding group exhibited degeneration. This is probably due to the mild dehydration observed in this group of chickens. Aside from this abnormality, all the other cells, and, by inference, kidney functions, seem unaffected.

As with the kidney, a variety of reasons make the kidney a prime target organ for toxic compounds. The kidney is the main site of excretion of many drugs and metabolites, and is therefore exposed to a variety of potential toxins. Blood flow to the kidney is relatively high when compared to most other organs, and thus any drug or chemical in the systemic circulation will be delivered in relatively high amounts. Toxins are concentrated in the tubular urine due to reabsorption of water and salt from the glomerular filtrate (Rush & Hook, 1988).

Both the proximal and distal tubular cells in the kidneys from all the groups appear normal, ruling out the possibility of accumulation of toxic chemicals from the proximal tubule by the tubular cells. The glomeruli from all the groups also appear normal.

The spleens from the three feeding groups appear unaffected (Figure 6.12). The lymphoid tissue in the animals fed *Spirulina* appears normal, as do the number and morphology of the lymphocytes in all the feeding groups.

The intestines examined exhibit no morphological lesions in response to the *Spirulina* supplement (Figure 6.13). The intestine epithelial layer is very succeptable to metal contamination. However, no corrosive lesions are noticeable on the epithelial tissue layer, indicating no heavy metal toxic effects. The villi appear normal, and the smooth underlying muscle does not exhibit morphological variation when compared to that of the control groups. The goblet cells appear to be intact, and of normal size and number.

The histopathology study points to the fact that there are no significant adverse pathological effects in chickens, following ingestion of up to 50% effluent-grown *Spirulina* in the diets of chickens.

All the above evaluations; that of nucleic acid, pesticide and heavy metal contents, the favourable survival of brine shrimp larvae, and the nutritional and toxicological evaluation in chickens points to the preliminary safety for further feeding trials with tannery-effluent grown *Spirulina*.

CHAPTER 7

FEEDING *Spirulina* TO THE SOUTH AFRICAN ABALONE, *Haliotis midae*

Summary It was shown that the commercially significant South African abalone *H.midae* accepts an agar-bound artificial diet, which has implication in the intensive rearing of this high-priced aquaculture delicacy. Lower concentrations of effluent-grown *Spirulina* were found to adequately supplement the artificial fishmeal-based diet, resulting in favourably comparable growth kinetics.

7.1 INTRODUCTION

The genus *Haliotis* comprises a group of large marine gastropods, commonly known as the abalone, of about 100 known species (Hahn, 1989). They belong to the primitive order Archaeogastropoda, and fossils dating to the Oligocene period (30-50 million years) have been found (Cox, 1960).

Abalone are very highly prized for the delicately flavoured white meat of their large muscular foot (Chen, 1989). Although exploited by humans for many thousands of years, recent demand exceeds that of natural production from fisheries. This has led to the development of abalone farming techniques, which, in the past number of decades, has developed into a small but significant industry (Fallu, 1991).

7.1.1 Biology and Ecology

Abalones are ubiquitous along the rocky shores of all the major continents and islands of the Pacific, Atlantic and Indian Oceans (Cox, 1960), with commercially significant species found in the temperate waters off California, Japan, South Australia, New Zealand and South Africa (Fallu, 1991).

Abalone have a large muscular foot that fills the ventral portion of the shell, and is used for locomotion and adhesion to the substrate. The foot is covered by the characteristic auriform shell, which is perforated by a row of respiratory pores that also function as excurrent channels for excretion and gamete release (Cox, 1962).

The head is attached to the anterior portion of the foot, and bears two stalked eyes and two retractable sensory cephalic tentacles. The edge of the foot is surrounded by a ruffled flap of tactile sensory tissue, the epipodium, which bears short tentacles used for chemical and tactile sensing (Cox, 1962).

Abalone are herbivorous (Cox, 1962) and feed at night (Wells & Keesing, 1989).

7.1.2 Commercial Exploitation

Approximately 15 abalone species are exploited commercially (Hooker & Morse, 1985). In 1989, the abalone fishery harvest from the various producing countries have been estimated at 5000 tons (T) (Australia), 5000 T (Japan), 1147 T (New Zealand), 850 T (Mexico), 625 T (South Africa), 300 T (California), and 73 T (Canada & Alaska) (Britz, 1990). These are generally cold water species. Most abalone fisheries have collapsed, the only noteworthy survivors left are based in Australia and Japan.

Common species that are exploited commercially are the blacklip abalone, *H.rubra* (Australia), the red abalone, *H.rufescens* (California and Mexico), the ezo abalone, *H.discus hannai* (Japan), and the paua, *H.iris* (New Zealand). The perlemoen abalone, *H.midae*, is the favoured species in South Africa (Fallu, 1991).

Although many of the wild fisheries have collapsed, there is still a strong demand for abalone. If abalones can be produced by farming at a price that matches the demand, there is scope for an industry (Fallu, 1991).

Abalones have many virtues that make them ideal for intensive farming. They can be bred (Peter Britz, pers comm.), their larval time is short (Fallu, 1991), and they accept formulated

feeds (Uki *et al*, 1985b). They do not waste food energy in swimming or thermoregulation. They are generalist herbivorous, and do not necessarily require expensive animal protein (Fallu, 1991).

Abalone are marketed in a variety of forms. These include live abalone, frozen whole abalone, meats, canned abalone, cocktail and dried abalone meat. Also of marketable value are the shells (notably the New Zealand paua), and abalone seed (Fallu, 1991). Retail prices for abalone meat were estimated at $35-40/pound in 1989 (San Franscisco Examiner, 1989).

7.1.3 Commercial Abalone Farming in South Africa

Six species of abalone are found in South Africa (Kilburn & Rippey, 1982), but only *H.midae* is fished commercially (Barkai & Griffiths, 1986). Abalone are a potential prime mariculture species for South Africa due to the lucrative export market that exists for the product, and because environmental conditions along the coast are ideal for abalone culture (Britz, 1990). *H.midae*, the perlemoen abalone, is distributed from St. Helena Bay on the west coast, to the southern Transkei coast along the east coast of Southern Africa (Day, 1974).

The commercial exploitation of the South African perlemoen was initiated in the early 1950's. The export market grew rapidly, especially to Japan and SE Asia (Dixon, 1992). Harvesting of the abalone is concentrated between Cape Hangklip and Cape Agulhas. When production peaked in the mid-1950's, control measures were initiated. Size limits were introduced in 1953, as were the introduction of permits to restrict free-diving harvests. Freezing of abalone was introduced in 1963, and led to an even greater exploitation of the natural population. Production quotas were introduced in the same year. Over-exploitation of natural stocks of abalone has resulted in a substantial decrease in production, dropping from 810 MT whole mass in 1976, to 629 MT in 1991 (Dixon, 1992). This figure represents the wild-harvested stocks, and restriction quotas are based on research carried out by the Sea Fisheries Research Institute, Cape Town, South Africa (McVeigh, 1991)

The decline of production yields, coupled with the escalating price, have stimulated interest in abalone aquaculture for the enhancement of natural stocks by reseeding, thus providing a direct supply to meet the market demand (Morse, 1990).

South Africa, led by the Department of Ichthyology and Fisheries Science, Rhodes University, Grahamstown, is currently devising appropriate technology for the aquaculture production of the perlemoen abalone. The University of Cape Town, The Sea Fisheries Institute, and the CSIR in Stellenbosch are also currently involved in applied research in this field. Much of the technology for abalone culture has already been developed elsewhere, and thus its establishment in South Africa is largely a matter of technology transfer and adaptation to local conditions (Britz, 1989).

The main problems facing the cultured abalone industry in South Africa include the lack of market research, unwieldy legislation, lack of permit application procedures, abalone seed supply, potential yield of available feed plants, and available coastal sites for establishing commercial abalone farms (McVeigh, 1991).

7.1.4 Abalone Aquaculture

The economic viability of abalone culture depends, amongst others, on the growth rate and feed conversion efficiency of the species (Hahn, 1989). Settled juvenile abalone feed on diatoms, later changing their diet to macroalgae. Abalone are generalist herbivores, and feed on a wide range of algae, although preferences have been noted. The kelp, *Ecklonia maxima*, and the red macroalgae, *Plocamium corallorhiza*, have been demonstrated to be the main algal species consumed by *H.midae* (Barkai & Griffiths, 1986).

Abalone have slow growth rates, taking about 14 years to reach the legal size limit. This makes the rearing of abalone to their legal fishery size impractical, and to reduce culture time, abalone are harvested as "cocktail-sized" animals when they are 2.5-5 years old (Hooker & Morse, 1985).

Two options are available for the exploitation of abalone. The first, ocean ranching, requires the release of abalone spat into a natural habitat, where they are left to grow to marketable size. This method does not require high running costs, but losses due to predation and poaching could be high (Saito, 1984).

The second option involves spawning and rearing to marketable size. Juveniles may be kept in shore-based tanks, which require high investment and operation costs, or they may be reared in containment cages in sheltered waters. Disadvantages associated with this option include feeding difficulties, continual maintenance, and storm-induced equipment damage (Hooker & Morse, 1985).

7.1.5 Artificial Diets for Abalone

Available diets include harvested algae (Uki *et al.*, 1986b), cultured algae, and formulated feeds (Fallu, 1991). It is believed that the development of an abalone industry relies on the development of a viable, cost-efficient formulated feed (Fallu, 1991). The Japanese abalone industry were the first to devise artificial diets which allowed for on-shore cultivation (Hahn, 1989). Advantages of artificial diets include ease of storage, lower costs than frozen or dried macroalgae, the facilitation of the use of automatic feeders (Hahn, 1989), and better growth rates (Uki *et al.*, 1985b; Nie *et al.*, 1986). Because artificial diets provide the abalone farmer with a reliable and convenient food source with a consistent nutrient quality, it seems that the future of the industry would come to rely on artificial diets as the main feed source (Fallu, 1991).

Research into the nutrition of abalone has centred on the evaluation of various ingredients in artificial diets (Uki *et al.*, 1985a; 1985b; 1986a; 1986c; Uki & Watanabe, 1986; Ogino & Ohta, 1963). Optimum protein levels are between 20 and 30% (Ogino & Kato, 1964; Uki *et al.*, 1986a). This protein requirement is high for a naturally herbivorous animal. Superficially, it seems that fishmeal is the most desirable form of protein (Fallu, 1991). There is also evidence that casein as a protein source results in higher growth rates than those animals fed fishmeal. Casein, however, is more expensive, and it would not be economically viable to include this purified protein in abalone diets (Fallu, 1991).

The optimum lipid level is 5% (Uki *et al.*, 1986c) or less (Fallu, 1991). Whilst lipid is generally more expensive than carbohydrate, a given amount of lipid is generally a better source of energy than the same weight of carbohydrate (Fallu, 1991). A lipid source containing 1% omega-3-lipids has been found to be satisfactory for enhanced growth (Uki *et al.*, 1986c).

Exact composition of the carbohydrate source is not important. Some food formulations utilise starch or dextrin as a carbohydrate source. They may constitute 5-30% of the total diet. Many diets have binders that have an added function as a source of carbohydrates (Fallu, 1991).

Minerals and trace elements are supplemented to a level of 5% (Uki *et al.*, 1985a).

The state of knowledge of abalone nutrition is still very rudimentary, with the main constraint being the development of an artificial diet with which to test the quantity and quality of essential ingredients (Simons, 1990; Hecht & Britz, 1990).

7.1.6 Economics of Feed Production

Feed costs constitute the bulk of the operating costs in intensive aquaculture (Brown *et al.*, 1989). It is thus essential that artificial diets supply the correct nutrient concentration in a readily available form to enable optimum utilization of nutrients (Hastings & Dickie, 1972).

The most probable natural algal diet for South African conditions would utilise the giant kelp, *E.maxima*. However, the production costs of kelp as a diet for *H.midae* are estimated to be higher than those associated with formulated feeds (Dixon, 1992). A formulated feed with fishmeal as the main protein source has an estimated cost of R 2500.ton^{-1} dry weight, compared to R 400/ton for wet kelp. However, when the respective feed conversion ratios (FCR) are considered, the final production costs of using kelp are more expensive. A fishmeal-based dry pellet and wet kelp have FCR's of 1.2:1 and 11:1, respectively. Thus, ten times more kelp is required to produce the same growth rates as those obtained with a fishmeal-based diet. The production costs using kelp will thus be 1.5 times higher than those

where an artificial fishmeal-based diet is used (Dixon, 1992). In conjunction with this, the growth rates of *H.midae* are higher on an artificial fishmeal diet than on a wet kelp diet (Peter Britz, unpubl.).

7.2 RESEARCH OBJECTIVES

Due to the current interest in the intensive rearing of the South African abalone *H.midae*, the Department of Ichthyology and Fisheries Science, Rhodes University, Grahamstown, has initiated studies on the effectivity of an artificial diet for abalone nutrition. To date, current work has centred on the use of fishmeal as the main protein source. However, due to the continually escalating costs of fishmeal, the known nutritional value of *Spirulina* (discussed in Chapter 1), and the relatively low production costs associated with tannery effluent-generated *Spirulina* it was decided to undertake a study of the potential of this source of protein in the artificial diet rearing of the perlemoen abalone, *H.midae*. The following research objectives were identified:

1. Does *H.midae accept* an agar-based artificial diet?

2. Does *H.midae* accept an artificial diet supplemented with effluent-grown *Spirulina*?

3. Does *Spirulina* affect the growth rates of juvenile *H.midae*?

4. To what degree can *Spirulina* replace fishmeal in the artificial diet without adversely affecting growth rates?

7.3 MATERIALS AND METHODS

7.3.1 Experimental Animals

Juvenile *H.midae* (size range: 11.5 - 36.7mm; 0.59 - 6.90g) were collected from rocky outcrops at Port Alfred (33°35'S 26°52'E) along the East Cape coast of South Africa. The

abalone were transported back to the laboratory in Grahamstown in oxygen-filled bins filled with wet sea-weed, and were held in a recirculating system for two weeks prior to use.

7.3.2 Experimental Conditions

The experimental recirculating system used consisted of a 8000 L capacity header tank, a series of experimental holding tanks (total capacity of 900 L), and a series of four biological filters (total capacity of 4100 L). Water was pumped from the biological filters to the header tanks, from which it was gravity fed to the holding tanks.

Water temperature was maintained at 17-19°C using a heating coil and chiller unit in the header tank. Back-up temperature control was provided by the temperature control unit of the constant environment room in which the holding tanks were kept.

Salinity was maintained at 35 ppm by addition of deionised water to compensate for evaporative loss.

Lighting was supplied by 20 W Lascon Lighting Biolux fluorescent tubes, producing 1100 lumin, with a 97 RA index and a Kelvin rating of 6500 K. The photoperiod was maintained at 16L/8D cycles.

All experiments were conducted in plastic aquaria covered by fine-mesh shade-cloth.

7.3.3 Artificial Diets

The experimental diet formulations were based on the semi-purified diet developed by Uki *et al.* (1985a), and all contained approximately 30% protein and 5% fat. The compositions are shown in Table 7.1. Diets containing casein and fishmeal as a protein source were included as controls. Initially the degree of *Spirulina* supplementation was calculated as % of protein, the 100% diet being 100% of the protein provided by *Spirulina.* The protein content of *Spirulina* for the formulation of these diets was extrapolated from published protein values. An average of 65% protein (dry weight) was assumed. Subsequent evaluation of

Spirulina biomass showed a protein content of 48.5%. By this stage, however, the dry ingredients of the diets had been prepared, and the feeding trial was in its third week of execution. It was thus decided to continue with the same formulation to maintain consistency. The actual protein content of the test diets are shown in the last row of Table 7.1

Table 7.1 Basal composition of the artificial diets. All values are in percentages.

	Casein	**Fish-meal**	**5% Spir**	**10% Spir**	**50% Spir**	**100% Spir**
Casein	31.7					
Fishmeal		44.2	42.1	39.1	22.1	
Dextrin	41.3	41.3	41.3	41.3	41.2	41.2
FA Oil	5	0.45	0.51	0.59	1.17	1.9
Min.Mix.	4	4	4	4	4	4
Vitamins	1	1	1	1	1	1
Ch.Cl	0.5	0.5	0.5	0.5	0.5	0.5
Agar	9	9	9	9	9	9
Spirulina			2.15	4.3	21.5	42.9
% Protein	30	30	29.2	28.4	21.8	13.5

A 75% solution of Choline chloride (Ch.Cl) was used, necessitating 0.75 g/100% diet.

The fat mixture was prepared from equal volumes of sunflower and fish oil.

The composition of the mineral and vitamin mixtures are shown in Tables 7.2 & 7.3.

Table 7.2 Composition of the mineral mixture (after Uki *et al.*, 1985a). All values in g.

Mineral Mixture		Trace Element Mixture	
NaCl	1.0	$ZnSO_4.7H_20$	35.3
$MgSO_4.7H_20$	15.0	$MnSO_4.4H_20$	16.2
$NaH_2OPO_4.2H_20$	25.0	$CuSO_4.5H_20$	3.1
KH_2PO_4	32.0	$CoCl_2.6H_20$	0.1
$Ca(H_2PO_4)_2.H_20$	20.0	KIO_3	0.3
Fe-citrate	2.5	Cellulose	45.0
Trace Element	1.0		
Ca-lactate	3.5		
TOTAL	100.0	TOTAL	100.0

Table 7.3 Composition of the vitamin mixture (after Uki *et al.*, 1985a). All values in mg, unless otherwise specified.

Thiamine HCl	6	Folic Acid	1.5
Riboflavin	5	PABA	20
Pyridoxine HCl	2	Menadione	4
Niacin	40	B_{12}	0.009
Ca Pantothenate	10	Ascorbic Acid	200
Inositol	200	Vitamin A	5000 IU
Biotin	0.6	Vitamin D	100 IU

The dry ingredients, with the exception of the agar, were premixed, homogenised in a hammer mill, and stored at 4°C until needed.

Diets were prepared by boiling the agar (1.5 %) for 5 mins and allowing the solution to cool to 40°C. The premixed ingredients were then stirred into the agar solution, and allowed to set in petri-dishes to a depth of 2-3 mm. The moist diet was cut into smaller sections, and these were used in the feeding trials. The addition of water to the ingredients increased the wet weight by a factor of seven.

7.3.4 Feeding Trials

Duplicate tanks, each holding 20 juvenile *H.midae*, were used for the evaluation of each test diet. Animals were measured in terms of length, individually weighed and marked using dymo tape, so as to follow the growth trend of individual animals. The abalone were allowed to acclimate for three days after marking, prior to the start of the feeding trials.

Animals were fed 3-4 g (wet mass) feed in the evening. The residual feed was collected again the following morning, and reweighed. The feed consumption.day^{-1} was recorded.

The mortality rates were recorded. More than 90% of the mortalities were recorded within the first 10 days of the feeding trial. On day 8 the diets were supplemented with 5% oxytetracycline, which resulted in significant reduction in mortality.

Feeding trials were run for 40 days, after which the animals were analyzed in terms of length and mass increase.

Analyses thus included:

Increase in length
Increase in mass
Feed Consumption.

The mass and length increase of each animal was expressed in terms of the specific growth rate (SGR), which was expressed in % increase.day^{-1}:

$$SGR = (\ln w_t - \ln w_o)/t \times 100, \quad \text{where}$$

w_t = final mass/length,
w_o = initial mass/length

Both mass and length SGR's were subjected to analysis of variance, as described previously.

The increase in mass was linked to the feed consumption in terms of the feed conversion ratio (FCR), which is the ratio of the feed consumed per unit weight gained.

Because the actual protein content of *Spirulina* was 16.5% lower than assumed, it was decided to calculate the Protein Efficiency ratios (PER) of the individual test diets. The PER

is a measure of the efficiency of the protein consumed as indicated by the body mass increase. The PER of the various diets were calculated as follows:

$$PER = \frac{\text{Mass Gained}}{\text{Protein Fed}}$$

7.4 RESULTS

The mortality levels of the test animals are shown in Figure 7.1.

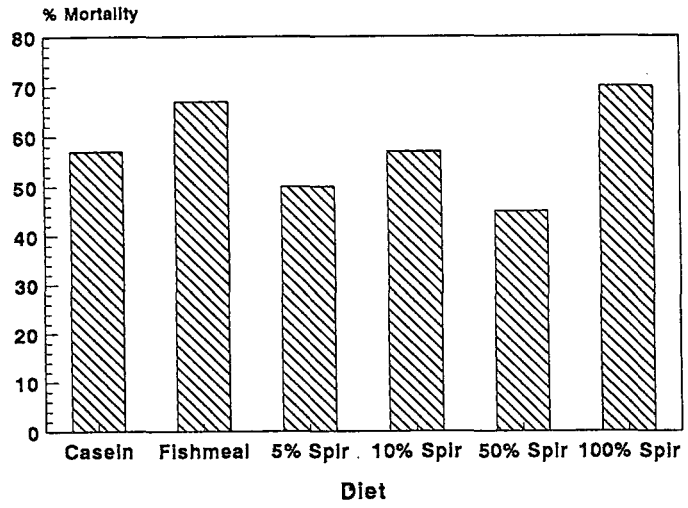

Figure 7.1 Mortality levels of *H.midae* during the feeding trial with various concentrations of *Spirulina*. More than 90% of the mortalities occurred within the first 10 days of the feeding trial.

The SGRs in terms of mass increase are shown in Figure 7.2.

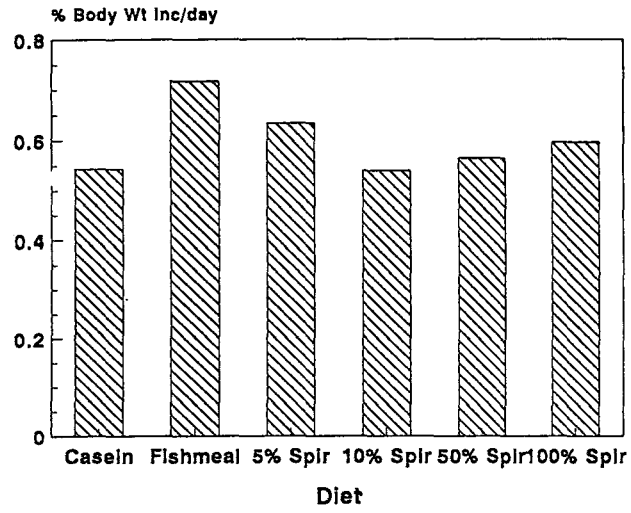

Figure 7.2 Specific growth rates of abalone fed a variety of test diets. Results are given in terms of % mass increase.day^{-1}.

As expected, the fishmeal-fed animals exhibited the best growth response in terms of mass increase. A 5% *Spirulina* supplement led to a SGR 16.8% greater than that of purified casein, albeit lower than the pure-fishmeal diet. There is then a concomitant increase in SGR with an increase in the concentration of *Spirulina*, all within the range of the casein diet.

The SGR in terms of length increase is shown in Figure 7.3.

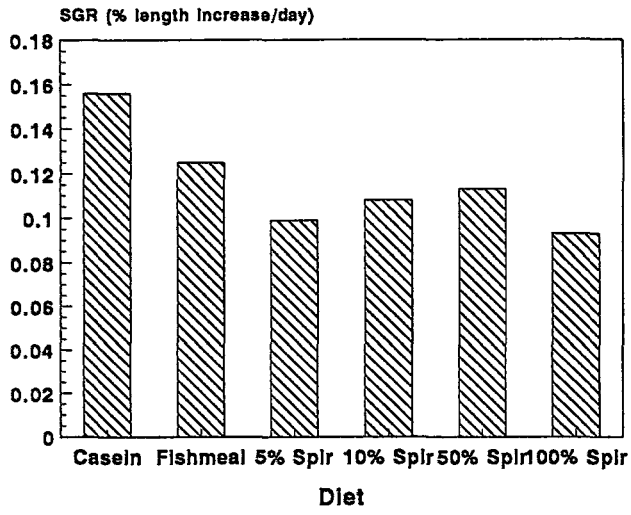

Figure 7.3 Specific growth rates of abalone fed a range of test diets. SGR is given in terms of % length increase.day^{-1}.

The casein-fed animals exhibited the best growth response in terms of length. The *Spirulina*-supplemented diets exhibited length increases similar to those of the fishmeal-fed animals.

The results of the analysis of variance tests are shown in Table 7.4.

Table 7.4 Analysis of variance of the relationship between degree of *Spirulina* supplementation and SGR's, in terms of mass and length.

SGR variable	Degrees of Freedom	F-ratio	p-value
Mass	5	0.799	0.5533
Length	5	6.459	0.0000

The mass variable shows the SGR to be independent of the degree of *Spirulina* supplementation in the artificial diet. The length variable, however, shows the SGR to be significantly related to the amount of *Spirulina* in the test diet.

The feed consumption is expressed in terms of % body weight consumed.day^{-1}, and is shown in Figure 7.4.

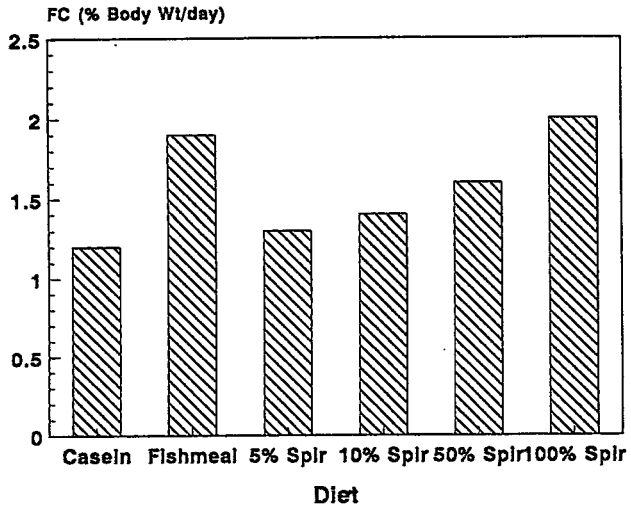

Figure 7.4 Feed consumption of abalone fed a variety of test diets. Results are expressed in terms of % body weight consumed.day^{-1}.

As expected, casein-fed animals expressed the lowest feed consumption, which can be attributed the purity of the protein. A 5% *Spirulina* supplement resulted in a feed consumption similar to that of purified casein. There is then a concomitant increase in feed consumption with an increase in supplementation of *Spirulina*. Fishmeal-fed animals exhibited a feed consumption similar to that of animals fed a 100% *Spirulina* diet.

The Feed Conversion Ratio (FCR) is the ratio of dry weight feed consumed per unit animal-mass increase. These ratios are shown graphically in Figure 7.5.

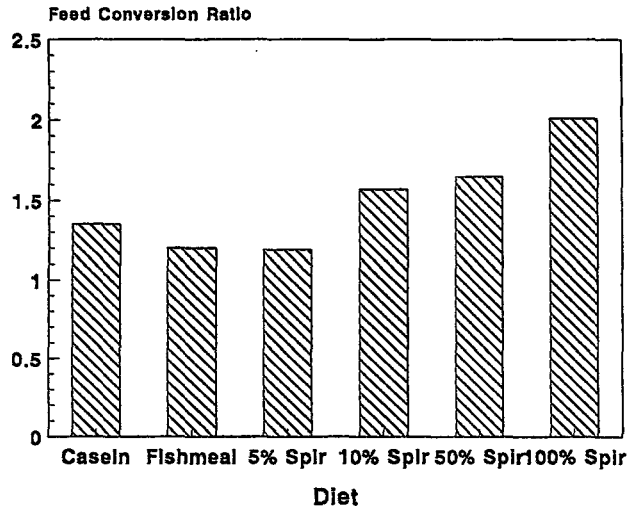

Figure 7.5 Feed conversion ratios of abalone fed a variety of test diets.

The greater the FCR, the less efficient the feed. Lower concentrations of *Spirulina* supplementation results in FCR's similar to purified casein and fishmeal diets.

The PER's are shown in Figure 7.6.

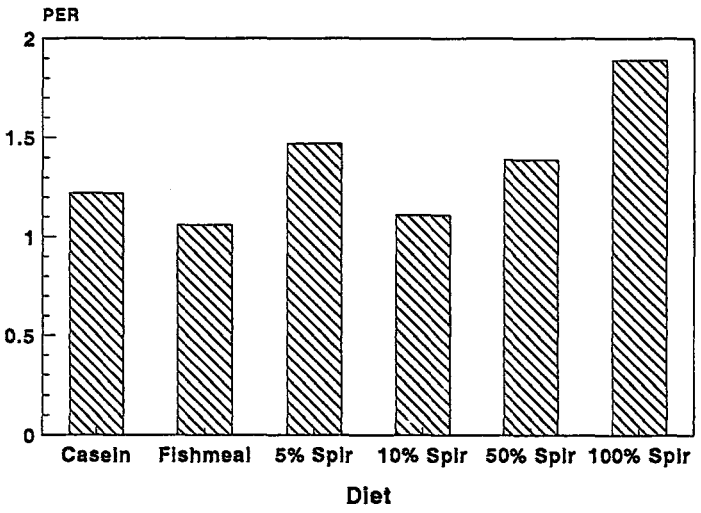

Figure 7.6 PER's of abalone fed a range of test diets.

The greater the PER, the greater the efficiency of the protein to elicit a growth response. Greater concentrations of *Spirulina* supplementation result in higher PER's.

7.5 DISCUSSION

7.5.1 Stress-Induced Mortality

Minimal mortality occurred following transfer of the experimental animals from their natural environment to the laboratory holding tanks. Drying of the shells and labelling of the animals in preparation for the feeding trial, however, resulted in significant mortality rates. A *Vibrio* infection was suspected. *Vibrio* is a gram-negative bacterium common in marine and estuarine environments, and on the surfaces and in the intestinal contents of marine animals (Krieg & Holt, 1984).

Semi-purified diets were then supplemented with 5% oxytetracycline during preparation of the hydrated diets. This antibiotic is active against gram-negative bacteria. The animals were treated with oxytetracycline for 5 days. Only 10% of the mortalities occurred after cessation of the antibiotic treatment, indicating that initial mortality was stress-induced due to preparative handling of the test animals. Low mortality rates following antibiotic treatment indicates that mortalities are unlikely to be diet related.

7.5.2 Artificial-Diet Acceptance

The results presented here are aimed as a contribution to the development of a cost-effective semi-purified artificial feed for the intensive rearing of *H.midae*. The first major conclusion that can be drawn from this study is that *H.midae* accepts the agar-based artificial feed. Protein leaching experiments (results not shown) indicate that minimal leaching of the protein occurs from the agar base into the surrounding sea-water after 24 hrs. Coupled with the ready acceptance of artificial feed by the abalone, it seemed feasible to use the agar-based artificial feed in nutritional studies. Studies by other authors have shown that the Japanese abalone *H.discus hannai* accepts Na-alginate-based artificial diets containing a variety of animal and plant protein sources without the alginate affecting the growth rates (Uki *et al.*,

1985a; 1985b; 1986a; 1986c; Uki & Watanabe, 1986; Ogino & Kato, 1964), and confirm the acceptability of the artificial diet exhibited by the test animals in this study.

The importance of the use of semi-purified diets in the development of formulated feed for specific aquaculture organisms has been stressed by a number of authors, and have been used for deletion and augmentation feeding trials to determine the optimum dietary requirements of the Japanese abalone *H.discus hannai* (Uki *et al.*, 1985b; 1986a; 1986c).

The acceptability of the artificial diet thus has important implications in terms of both nutritional studies and commercial application.

7.5.3 *Spirulina*-Supplemented Artificial Diets

The most accurate form of expressing size increase is the Specific Growth Rate (SGR), as this value linearises the initial size variation in the sample. The mass variable is usually preferred, because of the inherent degree of error in length measurements (Peter Britz, pers. comm.). The SGRs shown in Figure 7.2 indicate that the fishmeal-based diet elicits the best growth response in terms of mass increase. A 5% supplementation of *Spirulina* results in a SGR 12% lower than that of fishmeal, yet 14% higher than that exhibited by the casein-fed animals. The 10% *Spirulina*-supplement leads to a decrease in SGR (to a level similar to that exhibited by the casein-fed animals). There is then a concomitant increase in SGR with an increase in supplementation of *Spirulina*.

The FCRs (Figure 7.5) indicate that the fishmeal and 5% *Spirulina*-supplemented diets elicit the best responses in terms of protein conversion to body weight. Casein then produces the next highest efficiency, followed by decreasing efficiencies with a concomitant increase in supplementation with *Spirulina*. Analysis of variance indicates that degree of *Spirulina* supplementation does not significantly affect the mass increase. The length change, however, is dependent on *Spirulina* supplementation, greater concentrations leading to lower growth rates. The lower growth rates observed at higher concentrations of *Spirulina* seem to confirm the results of Uki *et al.* (1985a), who found that *H.discus hannai* is able to utilise animal protein derived from fishmeal more efficiently than plant protein sources.

This study indicates that *H.midae* is able to utilise fishmeal more efficiently than *Spirulina*, although a 5% supplement of Spirulina results in growth rates favourably comparable to a pure-fishmeal diet.

The increase in feed consumption with an increase in the degree of *Spirulina* supplement proved to be an interesting result. Three possible explanations can be presented to explain this.

1. The first centres on the possibility that the *Spirulina* protein is not readily available for digestion by the abalone. This may be due either to protein degradation during *Spirulina* processing or feed manufacture, or due to the inability of the abalone to efficiently process higher concentrations of biomass. This latter reason is unlikely as *Spirulina* does not possess an indigestible cellulose cell wall (Richmond, 1986b). It has been shown, however, that breaking the cell wall by thermal shock or ultrasonication renders large *Spirulina* ingestible and digestible for the brine shrimp *Artemia salina* (Sorgeloos, 1973). The herbivorous nature of the abalone also indicates that it is adept at plant-protein digestion, indicated by the long, complex alimentary tract (Campbell, 1965). It seems thus that there may be some protein breakdown during the course of *Spirulina* processing and feed manufacture. An evaluation of the effects of drying and processing indicate that some protein loss does indeed occur during processing of the biomass. It remains to be determined whether the process of feed manufacture results in protein loss, although this is unlikely, as low, non-denaturing temperatures are maintained throughout the entire feed-manufacture process.

2. The second explanation to account for increase in feed consumption with an increase in *Spirulina*-supplement centres on olfaction and chemoreception, which have been shown to be important in abalone feeding (Harada & Kawasaki, 1982; Harada *et al.*, 1987). The enhanced feeding response may thus be attributed to the leaching of feeding attractants from through the agar base (Santy Daya, pers. comm.). It has been shown that combinations of amino acids and lipids (Harada *et al.*, 1987), algal extracts (Sakata *et al.*, 1984) and purified algal compounds (Sakata & Ina, 1985)

evoke a chemoreceptive feeding response. *Spirulina* has also been linked in the appetite stimulation of the brine shrimp, a variety of fish and marine larvae, crustaceans, tropical fish, and silver salmon (Earthrise Farms, California). It thus may be possible that an appetite stimulant in *Spirulina* may account for the enhanced feed consumption at higher concentrations of *Spirulina*. This hypothesis may be tested by isolating various fractions from *Spirulina*, and using these extracts in feeding trials. This provides another potential area for applied research.

3. The third, and probable reason for an increased feed consumption with an increase in *Spirulina*, is the reduction in protein level with increasing amounts of the cyanobacterial biomass. As previously explained, this was due to diet formulation using extrapolated literature protein values.

   The animals fed low-protein diets need to consume more feed to maintain their optimum protein intake. This is accompanied by a higher feed conversion ratio, possibly due to the protein levels in the diet not being sufficient to maintain metabolic growth. This explanation is built on the assumption of a 30% protein optimum, as elucidated by Uki *et al.*, 1986a) for *H.discuss hannai*. The higher PER's at higher supplementations of *Spirulina* indicate that the lower protein levels are converted more efficiently to body mass. This could either be due to optimum protein levels for *H.midae* being lower than the 30% suggested by Uki *et al.* (1985a) for *H.discus hannai*, or the increased availability of *Spirulina* protein when compared to the fishmeal source.

The above results indicate that lower concentrations of tannery-effluent-grown *Spirulina* provide an adequate feed supplement in the artificial diet for the intensive rearing of *H.midae*. More research needs to conducted to determine the effectivity of different methods of processing of the biomass (fast drying, boiling, etc), so as to obtain a higher protein content. Research would need to centre on the improvement of protein quantity, quality and availability for the abalone, as well as isolation of the various lipids (mainly gamma-linolenic

acid and the omega-3 group of lipids) and growth factors which may have a positive effect on abalone growth rates.

Preliminary results do however indicate that effluent-grown *Spirulina* has potential as a feed supplement to fishmeal. This may contribute to the development of an economically viable artificial feed for the aquaculture of *H.midae*. Considering the relatively high cost of fishmeal in comparison to the very low algal-biomass production costs, this may have implications in the improvement of growth responses and capital saving for the intensive rearing of this high-cost aquaculture delicacy.

CHAPTER 8

FEEDING *Spirulina* TO THE RAINBOW TROUT, *Oncorhynchus mykiss*

Summary The feasibility of incorporating effluent-grown *Spirulina* in the artificial diets of rainbow trout was investigated. Results indicate that lower concentrations of *Spirulina* supplementation does not alter the growth rates, and there are no decisive pathological manifestations of toxicity. In addition, *Spirulina* supplements also result in desirable colour enhancement of both skin and flesh.

8.1 INTRODUCTION

8.1.1 Rainbow Trout Aquaculture

With the exception of the common carp, the rainbow trout (*Oncorhynchus mykiss*) is one of the most favoured aquaculture species. It is prized as a sport fish, has been harvested to produce thousands of tons of food, is farmed in many countries around the world, and is an extremely popular experimental fish (Gall & Crandell, 1992).

The rainbow trout is highly adaptable to its environment, which is one of the reasons it has achieved such wide distribution. Selective breeding in hatcheries is most likely the cause of the wide spawning period of the species. The natural habitat for most rainbow trout is fresh water at a temperature of about 12°C in the summertime. This species tolerates temperatures from 0-25°C, the optimum being 10-12°C (Gall & Crandell, 1992).

Two intensive aquaculture systems are presently in use for the commercial production of this food source. The production system employed is influenced mainly by the water quality. Readily available, fast-flowing fresh water at optimum temperature is conducive to raceway techniques, while large lakes and reservoirs are more adaptable to cage culture processes. The economics of rainbow trout production is influenced, to varying degrees, by a number of factors, including marketing strategy, location, operation size, technological organisation,

stocking density, and a range of chemical and environmental factors (Logan & Johnston, 1992).

Feed is the largest cost component in trout production (Cowey, 1992), frequently accounting for more than half the operating costs (Lovell, 1989, Logan & Johnston, 1992).

Obviously, fast growth rates are desirable, and growth rates are affected by a number of factors, including feeding rates, age, survival, and water temperature and quality (Logan & Johnston (1992).

Because of its tolerance to relatively high water temperatures and relatively low oxygen levels, and its fast growth rate, the rainbow trout is the preferred species for freshwater farming of table fish. In 1985, over 189 000 tonnes of rainbow trout were produced in Europe, 95% of the production being in fresh water. In Italy and Spain, aquifers provide the water supply, while in northern Europe, surface waters are commonly used. Many hatcheries in the USA produce rainbow trout to improve sport fisheries (Laird & Needham, 1988). In South Africa, rainbow trout are produced both commercially and to supplement the natural stocks (Hecht & Britz, 1990).

There are two major research areas of concern in the farming of rainbow trout. The first concerns the environmental and nutritional requirements for optimum growth. The second aspect concerns the final product quality.

Nutritional requirements of trout has been dealt with in some detail by a variety of researchers, and a review of their findings is given below.

Product quality has thus far been based almost entirely on gross fish mass, and, as such, has received little attention in terms of its effect on producer economics and consumer acceptance of the product. Two factors identified were level and type of pigmentation and fatty acid and lipid content of trout (Gall, 1992).

8.1.2 Nutritional Requirements

In their natural environment, rainbow trout feed mainly on invertebrates which are composed of 37-66% protein, 9-33% fats, 3-28% minerals, with the rest being carbohydrates. This composition gives a good indication of the dietary requirements of these trout and their ability to use different types of nutrients, and therefore alludes to the required composition of the manufactured diet used in intensive aquaculture (Billard, 1990).

The information at present indicates that trout, as with all other fish, have the same general needs in protein, fats and carbohydrates as other animal species. In systems of intensive aquaculture however, it is necessary to incorporate all the essential nutrients into the manufactured diets (Murai, 1992).

8.1.2.1 Proteins

Trout food characteristically contains high levels of proteins, and fishmeal is generally recommended as the first choice to supply needs. Investigations have shown that protein levels of 30-60% are desirable, although most authors recommend a protein level of 40-45% in the diet (Billard, 1990; Murai, 1992). Variations in the required levels of protein are influenced by factors such as age, water temperature, type of protein used, and the accompanying constituents of the diet. Starter feeds usually contain 38-55% protein, with a concomitant decrease in required protein with an increase in fish weight. The high levels of protein required are due, in part, to the fact that protein is used as an energy source. It is possible to decrease the amount of protein in the diet, but this must be accompanied by an increase in the carbohydrate or fat content (Billard, 1990).

One of the ultimate goals of protein nutrition in fish is the formulation of high quality, yet cost effective feeds, taking advantage of various protein sources as alternatives to fishmeal, which is expensive and of limited supply (Murai, 1992). The source of protein used in trout diets depends on the amino acid composition. The general amino acid requirements of salmonids have been quantitatively determined based on dose-response curves of weight gain in feeding studies. The requirements are shown in Table 8.1.

Table 8.1 Amino acid requirements of salmonids, in a diet composed of 40% protein (after Billard, 1990).

Amino acid	Requirements (% Of Dry Diet)	Requirements (% Of Protein)
Arginine	2.4	6.0
Histidine	0.7	1.8
Isoleucine	0.9	2.2
Leucine	1.6	3.9
Lysine	2.0	5.0
Methionine + Cystine	1.6	4.0
Phenylalanine + Tyrosine	2.1	5.1
Threonine	0.9	2.2
Tryptophan	0.2	0.5
Valine	1.3	3.2

Smaller fish require higher levels of arginine, lysine and tryptophan than do larger fish (Page & Andrews, 1973). It has been reported for several species, including rainbow trout, that protein requirements in fish decrease as they increase in size (Wilson & Halver, 1986). An increase in water temperature also leads to an increase in feeding activity, digestive and growth functions (Murai, 1992). Other factors influencing the required protein level in the diet include physiological function (whether the protein is required for maintenance or growth), protein quality, the availability of non-protein energy in the diet (in the form of fats or carbohydrates), feeding rate, the availability of natural foods, and the economics of feed production (Lovell, 1980).

The information available on the effects of amino acid supplementation are sometimes conflicting. In general, however, supplementation with sulphur amino acids (Murai, 1992), and especially methionine (Tacon & Jackson, 1985) is a common practice.

Fishmeal is the major dietary protein source because of its high nutritive value and palatability, and dietary content usually ranges from 25-65% of commercial salmonid feed (Murai, 1992). In general, fishmeal has excellent digestibility (85-95%). The addition of

large concentrations does not affect the protein digestibility. The digestibility of vegetable proteins is generally lower (60-80%) (Billard, 1990).

Numerous studies have been conducted on the utilization of alternative and less expensive protein sources, due to the high cost and short supply of good quality fishmeal (Murai, 1992). Exclusive dependence on fishmeal is thus likely to hamper further development and intensification of pisciculture. Unfortunately, attempts by feed manufacturers and fish nutritionalists to replace the fishmeal component of practical fish feeds with alternative sources of protein have generally resulted in reduced feed efficiency and growth (Alexis *et al.*, 1985; Andrews & Page, 1974; Koyama *et al.*, 1966; Murai, 1992; Tacon & Jackson, 1985). Meat and bone meal, blood meal, poultry by-products, dried yeast, hydrolysed feather meal and corn gluten meal are commonly incorporated at low levels as secondary protein sources in practical fish feeds (Murai, 1992).

Soybean meal appears to be efficiently utilised by most fish species, although replacement of fishmeal by soybean meal has resulted in reduced growth rates of rainbow trout, when compared to fishmeal-fed animals (Reichle, 1980; Dabrowski *et al.*, 1989). However, heat-treated soybean meal can be used as a major protein source for grow-out rainbow trout if essential amino acids are supplied (Murai, 1992).

It was also found that higher levels of protein were required for maximum growth rates in all-plant diets than in diets containing some fishmeal. Growth rates improve as fishmeal is supplemented to the plant protein source. This can be attributed to the methionine deficiency present in plant protein. The benefit of adding fishmeal diminishes as the amount of fishmeal or percentage of protein increases in the diet, which indicates that the fish's requirement for methionine could be met by feeding either more (plant) protein than is required, or by feeding more fishmeal (Lovell, 1980).

8.1.2.2 Fats

In comparison to the poorly digested complex carbohydrates, lipids are a more important source of energy. Furthermore, fats are made up of essential fatty acids, fat-soluble vitamins,

and carotenoid pigments. The supply of fats is essential, and the use of fat-reduced diets leads to lowered growth rates and dermal lesions. Trout can use up to 25% fat without apparent damage (Billard, 1990).

For trout, as with most fish, the essential fatty acids belong to the *n*-3 series, the most simple of which is linolenic acid. The requirement of linolenic acid in trout diets was found by Castell *et al.* (1972) to amount to 1% of the dry diet. This value was later amended to 0.8-1.7% when the diet contained 5% lipid (Watanabe *et al.*, 1974). It is known that an excess of linoleic acid (series 6 fatty acid) brings about growth depression (Billard, 1990). Other *n*-3 fatty acids with a greater degree of unsaturation, such as ecosapentanoic acid and decosahexanoic acid are twice as effective as linolenic acid, thus it is necessary to incorporate these only to a level of 0.3-0.5% in the diet (Billard, 1990).

8.1.2.3 Carbohydrates

In nature, the diet of the trout is practically totally devoid of carbohydrates except for chitin (which they have little ability to digest). In fact, fish are poorly adapted to utilise simple sugars such as glucose and sucrose, and it has been shown that features of intolerance appear when there is more than 20% of such sugars in the diet.

The only carbohydrates economically suitable for inclusion in commercial diets are those of complex structure, such as starch from cereals, roots and tubers. The efficiency of digestion of these carbohydrates increases when the molecular complexity decreases (Billard, 1990).

8.1.3 Feeding Algae to Fish

As previously mentioned, the final quality of fish in terms of consumer acceptance and producer economics is an aspect of aquaculture production that has been neglected in the past (Gall, 1992). Two main criteria are important here: nutritive quality, and aesthetic quality. Nutritive quality has been dealt with insofar as the nutritional requirements of the fish, and final product constitution (in terms of protein, fat, etc.) as a result of different feed sources has been neglected.

Aesthetic quality is another aspect that has been dealt with only superficially (Gall, 1992). The main area of concern here is the pigmentation of the cultured fish. Although much research has been conducted on the pure science of colour enhancement, work has centred mainly on the elucidation of the biochemical pathways involved, and economics has centred on the beneficial properties of colour enhancement in ornamental fish. The economic manifestation of colour enhancement in table fish has not received an appropriate amount of attention.

8.1.3.1 Nutritional Considerations

To penetrate the animal feed market and be economically viable, algae must compete with equivalent feed ingredients, mainly fishmeal and oilseed meals (soya, etc). Algae that have thus far been used as animal feeds include species of green, blue-green and pigmented flagellates, the advantage being that artificially cultivated algae are highly effective producers of protein as far as use of land and water resources are concerned (Berend *et al.*, 1980).

The proposal for the utilization of microalgae as a potential protein source in fish has been investigated by a number of authors (Ahmad, 1966; Gupta & Ahmad, 1966; Matty & Smith, 1976; Reed *et al.*, 1974; Sandbank & Hepher,1980; Stanley & Jones, 1976) These trials have been conducted on small samples in aquaria or tanks. Most of the experiments showed favourable results, the fish being able to effectively utilise lower concentrations of the algae. The main drawback regarding the use of microalgae as fish feed has been the exorbitant production and processing costs (Sandbank & Hepher, 1980).

Cultured microalgae have been fed directly (alive) to the consumer, and they have also been used successfully in a concentrated or preserved form. In particular, pure-culture-grown *Spirulina* has been used successfully in the culture of penaeid shrimp larvae, bivalve mollusc larvae, brine shrimp, marine rotifers (de Pauw & Persoone, 1988), koi carp, black tiger prawns, *Daphnia*, tropical fish, *Ayu* (a freshwater sushi fish), yellowtail tuna, silver salmon, catfish, sturgeon and blue lobster (Earthrise, California). *Spirulina* has also been used successfully in the culture of *Tilapia rendalli* and *Cyprinus carpio specularis*, growth rates

increasing by 62.5% and 100% respectively. Carp also exhibited enhanced growth rates when fed *Spirulina* (Ehrenberg, 1980).

Although the algal market was initiated on the chlorophyte *Chlorella*, it is gradually giving way to the cyanophyte *Spirulina* (Ehrenberg, 1980), the nutritional virtues of which are expounded upon in the preceding chapters.

8.1.3.2 Colour Enhancement by *Spirulina*

In the past, synthetically produced pigment (notably astaxanthin and canthaxanthin) has been used for the intensification of fish colour. However, due to the high production costs and consumer preference for natural colourants, these dyes are becoming less popular (Hardy *et al.*, 1990). Furthermore, the use of artificial pigments in feeds is accepted in some parts of the world but not in others, which has a complicating effect on the market (Gall, 1992).

Alternatives to synthetic colourants for rainbow trout have also been investigated, including marigold petals, shrimp head meal, tumeric (Boonyaratpalin & Unprasert, 1989), *Adonis aestivalis* and paprika. Although diets supplemented with *Adonis* does lead to enhanced colouration, the presence of alkaloids restricts the use of this flower as a pigment source (Kamata *et al.*, 1990). Plant pigments also produce a yellow flesh colouration due to the lack of red xanthophylls, while crustaceans have a low digestibility and an undesirable high mineral content (Kamata *et al.*, 1990). *Phaffia rhodozyma*, a red yeast, has also been used with some success in the colouration of rainbow trout, but the costs involved in cell wall rupture make the use of this organism economically unfeasible (Gentles & Haard, 1991).

Because of the high concentration of pigments, *Spirulina* is used extensively by the Japanese for the colouring of koi and other ornamental fish. It has also been found to improve the colour of trout, salmon and shrimp. The pigments are highly concentrated and converted more efficiently than some synthetic pigments. In fact, Israeli market studies have showed consumer preference for algae-fed fish based on appearance (Ehrenberg, 1980).

The colour-enhancement potential of *Spirulina* can be attributed to the high concentrations of pigments, including β-carotene, echineonine, cryptoxanthin, zeaxanthin, myxoxanthphyll and oscillaxanthin (Richmond, 1986b).

Spirulina maxima fed to sweet smelt, *Plecoglossus altivelis* (Mori *et al.*, 1987; Henson, 1990), the red tilapia *Oreochromis niloticus* (Boonyaratpalin & Unprasert, 1989) and the striped jack, *Caranx delicatissmus* (Okada *et al.*, 1991) has resulted in significant enhancement of both flesh and skin colouration. This greatly enhances the economic returns, and it has been noted by Henson (1990) that sweet smelt fed *Spirulina* sells for almost double that of ordinary fish. He found that supplementation of the feed with *Spirulina* produces a superior product, reduces fry mortality and medication expenses. Although supplementation is expensive, the cost/performance ratio is favourable.

8.1.3.3 Toxicological Considerations

Evidence for the nutritional quality is only one of the basic requirements for successful utilization of algal biomass in feed and food preparations. Equally important is proof of the toxicological safety of the material. Since algae represent a new and unconventional protein source, detailed toxicological investigations have to be performed before the alga can be commercialised (Becker, 1988).

Although man is the target species for animal products, it cannot be assumed that the lower organism will act as a filter for toxins in the animal feed (van der Wal, 1980). Research on the toxicological effects of sewage-grown algae in the diets of fish, however, has shown that the replacement of fishmeal by algae (up to 25%) has no detrimental effects (Berend *et al.*, 1980; Becker, 1988).

8.2 RESEARCH OBJECTIVES

The feasible application of tannery effluent-grown *Spirulina* in the culture of rainbow trout can only be considered if the following questions are investigated:

1. Do rainbow trout accept pellets containing effluent-grown *Spirulina*?

2. Does the addition of effluent-grown of *Spirulina* affect feed consumption and growth rates when compared to that of fishmeal-reared animals, and to what level is the supplementation of *Spirulina* beneficial to the physiology and economics of the process?

3. Does the addition of effluent-grown *Spirulina* result in useful colour enhancement of the skin and flesh?

4. Are there any toxicological constraints on the use of effluent-grown *Spirulina* in the nutrition of rainbow trout?

8.3 MATERIALS AND METHODS

8.3.1 Experimental Animals

Juvenile rainbow trout (*O.mykiss*) hatched in the Department of Ichthyology and Fisheries Science, Rhodes University, were used in the feeding trials. All animals used were from the same hatched brood, and were of uniform size and weight.

8.3.2 Experimental Conditions

Juvenile rainbow trout were held in a recirculating hatchery system, which consisted of 3 header tanks (total capacity = 27 000 L), a series of conical, fibreglass holding tanks (capacity of 70 L each), a sedimentation tank (capacity = 10 000 L), and a biological filter (capacity = 4 500 L). The biological filter employed a volume exchange rate of 30 min, and the culture tanks exhibited an exchange rate of 15 min/volume change/tank. This is within the culture requirements laid out by Logan & Johnston (1992).

The experimental system is shown in Figure 8.1. Water was pumped from the biological filters to the header tank, from which it was gravity fed to the holding tanks. The conical

shape of the holding tanks facilitated cleaning by siphoning, which was performed every week. A central surface-level drainage overflow pipe was erected in the centre of each tank. Water flowed into the overflow pipe, into a collecting gutter, and was then fed to the filter, and recirculated. Evaporative loss from the system was compensated for by refilling of the header tank.

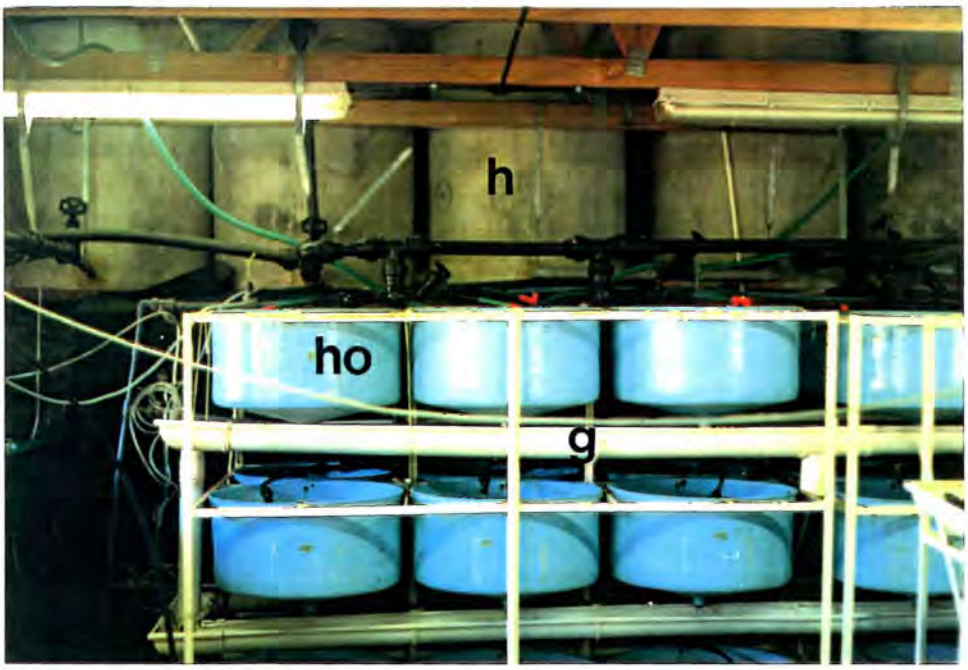

Figure 8.1 Experimental recirculating system for the housing of juvenile rainbow trout. **ho** = holding tank; **h** = header tank; **g** = gutter to biological filter. The biological filter is housed in a separate outdoor unit, and is not shown in the figure.

Water temperatures were maintained at 13-15°C by a separately-housed temperature control unit, utilising a heat-exchange series of elements.

8.3.3 Artificial Diets

Artificial diets were formulated according to the dietary requirements for trout fingerlings suggested by Halver (1989). All the diets were formulated to contain 40-42% protein and 14% lipid. The basal composition of the diets is shown in Table 8.2.

Table 8.2 Composition of the semi-purified artificial diets used in feeding trials with juvenile rainbow trout. All values are in %.

	Fishmeal	**5% *Spir.***	**20% *Spir.***	**53% *Spir.***
Wheat	30.7	30.8	32.2	35.7
Fishmeal	60.9	55.1	37.9	
FA Oil	7.5	7.7	8.3	9.8
Spirulina		5.0	20.0	53.1
Vitamins	0.5	0.5	0.5	0.5
Min.Mix.	1.0	1	1	1
Ch.Cl	0.5	0.5	0.5	0.5
Carotenes from *Spir.*		0.023	0.092	0.243

The vitamin and mineral mixtures were made according to the formulations of Tables 7.2 and 7.3.

The amino acid profiles of the protein dietary components are shown in Table 8.3.

Table 8.3 Essential and semi-essential amino acid profiles of fishmeal (Lovell, 1989) and *Spirulina* used in the various test diets. Amino acid concentrations are given in % of protein. (N.D. = not determined)

	Amino-acid/protein (%)	
Amino acid	**Fishmeal**	***Spirulina***
Arg	6.1	2.5
His	2.4	0.7
Iso	4.7	2.9
Leu	7.3	4.5
Lys	7.7	2.3
Met	2.9	1.0
Cys	0.9	0.0
Phe	4.0	1.8
Tyr	3.2	1.4
Thr	1.0	2.7
Try	1.1	N.D.
Val	5.3	3.7

As shown in Table 8.3, the amino acid profiles of fishmeal and effluent-grown *Spirulina* differ vastly, which would probably limit the degree of nutritionally useful *Spirulina* supplementation without additional amino acid supplementation.

Table 8.4 shows the derived amino acid contents of the various test diets.

Table 8.4: Essential and semi-essential amino acid contents of the various test diets. Compositions are calculated from the amino acid profiles (Table 8.2) and the percentage of the various ingredients used in the diets. It is assumed that all protein originates from fishmeal and/or *Spirulina*. Values are given in % of the dry mass. Requirements for trout were extrapolated from Hepher (1988), and the amino acid concentrations for fishmeal were taken from Lovell (1989).

Amino acid	Rainbow Trout Require-ment	Diet			
		Fishmeal	5% *Spir.*	20% *Spir.*	53% *Spir.*
Arg	1.24	2.49	2.32	1.77	0.58
His	0.56	0.96	0.89	0.66	0.16
Iso	0.84	1.92	1.81	1.46	0.70
Leu	1.56	2.98	2.80	2.26	1.06
Lys	1.88	3.13	2.89	2.15	0.54
Met	0.64	1.16	1.07	0.81	0.23
Cys	0.32	0.37	0.34*	0.23*	
Phe	1.12	1.64	1.52	1.18	0.42
Tyr	0.76	1.29	1.20	0.93	0.34
Thr	1.20	1.66	1.57	1.28	0.63
Try	0.16	0.43	0.39#	0.27#	N.D.
Val	1.12	2.14	2.02	0.69	0.88

* Only from fishmeal
N.D. Not Determined
\# *Spirulina* value N.D. Only the fishmeal contribution is shown

The first three diets satisfy the amino acid requirements of rainbow trout. The 53% *Spirulina*-supplemented diet, however, is deficient in almost all the essential amino acids.

The dry ingredients were mixed and homogenised in a hammer mill. Water was then added to give a crumble-consistency, and dried under a stream of cool air for 24 hrs. The resulting dry pellets were then sieved through a 715 μm sieve to give a uniform-sized pellet for all the diets. This is within the pellet size suggested by Halver (1989) for fingerling trout. The dried diets were stored in sealed jars at 4°C until needed. Figure 8.2 shows the prepared dry artificial diets.

Figure 8.2 Dry artificial diets for rainbow trout. **a** = Fishmeal diet; **b** = 5% *Spirulina*; **c** = 20% *Spirulina*; **d** = 53% *Spirulina*.

8.3.4 Feeding Trials

Duplicate tanks were prepared for the evaluation of each diet. Fish were individually weighed, and each tank contained 30 juvenile trout (mean weight/fish = 2.76 g ; SD = 1.08). Feeding rates were calculated from the levels suggested for fingerling salmonids by Halver (1989). For the first 4 weeks, fish were fed an averaged 5.5% body weight.day^{-1}, as the weight differences between the means from the different tanks were minimal. A 5.5% body weight feed necessitated a feeding of 4.7 g/tank/day. Fish were fed 5 times per day for the first 4 weeks.

Mortality rates were high during the first week of the feeding trial. Treatment with 5% oxytetracycline for 5 days resulted in a significant reduction in mortality.

Fish were individually weighed at the end of the 4 week period.

The feeding amount was then recalculated according to the 4-week weights. All fish were within the same size range, and their feed was reduced according to Halver (1989) to 3.9%

of the body weight. The feeding rate was reduced to three times per day, and the feeding trial continued for another 4 weeks.

At the end of the eight week period, fish were individually weighed.

Growth analyses were expressed in terms of:

Specific Growth Rate (SGR)
Feed Consumption
Feed Conversion Ratio (FCR)
Protein Efficiency Ratio (PER)

8.3.5 Histopathological Examination

At the end of the 8 week feeding trial, three fish from each feeding group were culled, and sections of the hearts, livers, kidneys, spleens and intestines were fixed with 10% formalin in Phosphate-buffered saline solution for later histopathological examination according to the procedure outlined in Chapter 6.

8.3.6 Pigment Extraction

The skin and underlying muscle was removed immediately upon culling, weighed, and transferred to dark vials containing dH_2O. Pigments were extracted according to a method modified from Gentles & Haard (1991). All procedures were performed in dim light so as to minimise photo-oxidation. Tissue was hydrated in dH_2O for 15 minutes. The water was discarded, and 20 ml 80% acetone was added to the vial. Tissues were homogenised for more than 10 minutes in a glass homogeniser. The resultant suspension was filtered through Whatman no. 1 filter paper, and the residue was re-extracted with two more solvent changes. The acetone residues containing the pigment extracts were pooled, and the volumes reduced under a stream of nitrogen. A spectrophotometric scan of the pigment extracts revealed an absorbance maximum at 474 nm. The absorbance of all the samples were read at 474 nm, and total carotenoids (TC) were determined using the Beer-Lambert law with an extinction

value (E) of 2100. Total carotenoid concentrations were converted to mg carotenoids.kg^{-1} flesh.

8.4 RESULTS

8.4.1 Mortality

The mean mortality levels are shown in Figure 8.3.

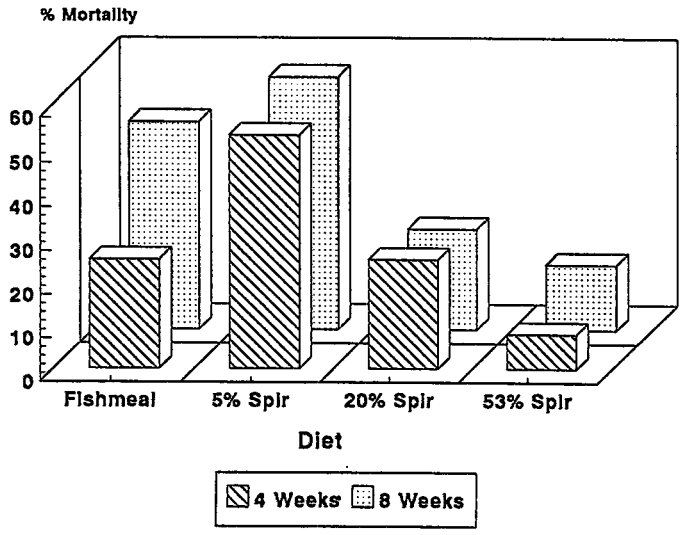

Figure 8.3 Mean mortality levels (%) of *O.mykiss* during the feeding trial with a range of test diets.

The mortality varies between 8 and 57%, with the highest mortality being experienced in the 53% *Spirulina*-supplemented feeding group. Mortalities dropped significantly following treatment with oxytetracycline.

8.4.2 Growth Responses

The growths rate in terms of the SGR (% body weight increase.day^{-1}) are shown in Figure 8.4.

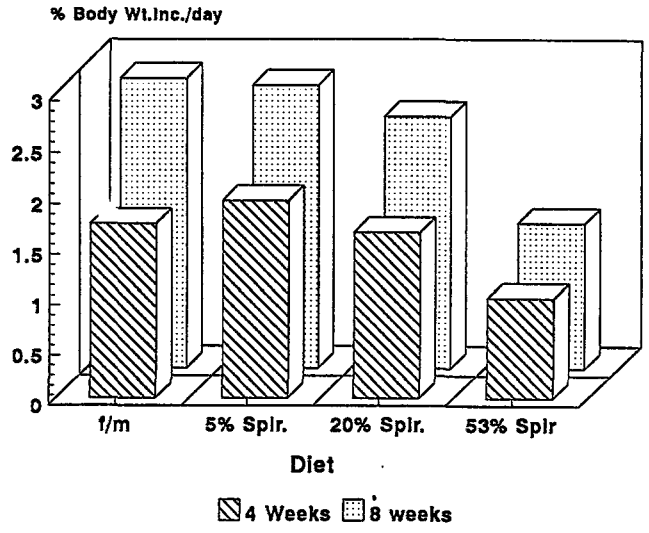

Figure 8.4 Specific growth rates of rainbow trout fed a variety of fishmeal-based artificial diets. SGRs were calculated at 4 and 8 weeks.

At 4 weeks, the animals fed a fishmeal diet supplemented with 5% *Spirulina* exhibited the best growth response. This is followed by the pure-fishmeal diet. There is then a concomitant decrease in SGR with an increase in *Spirulina* supplementation.

At 8 weeks, the animals fed a pure-fishmeal diet showed the best growth response. A 5% supplement of *Spirulina* resulted in a growth response similar to the fishmeal diet. There is then a concomitant decrease in SGR with an increase in *Spirulina* supplement.

The feed consumption, in terms of % body weight eaten.day^{-1}, are shown in Figure 8.5.

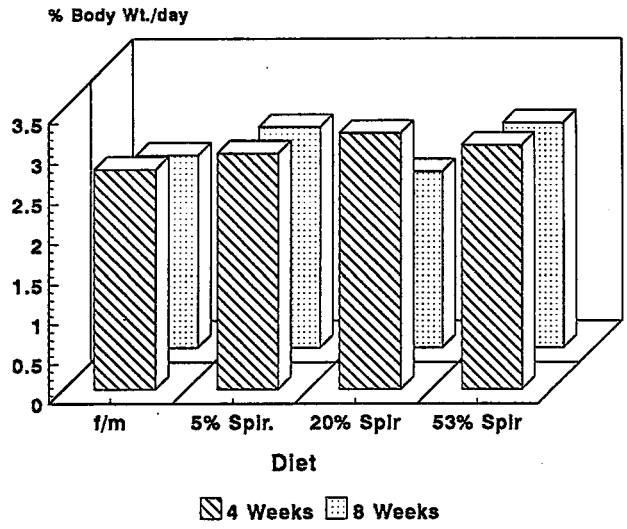

Figure 8.5 Feed consumption at 4 and 8 weeks of trout fed a range of artificial test diets.

The general trend is that of a lower feed consumption of the pure-fishmeal diet, with an increase in feed consumption with an increase in supplement of *Spirulina*. It must be stressed, however, that accurate analysis of the feed consumption is difficult if demand feeding is not practised, as pointed out by Halver (1989).

The FCRs are shown in Figure 8.6.

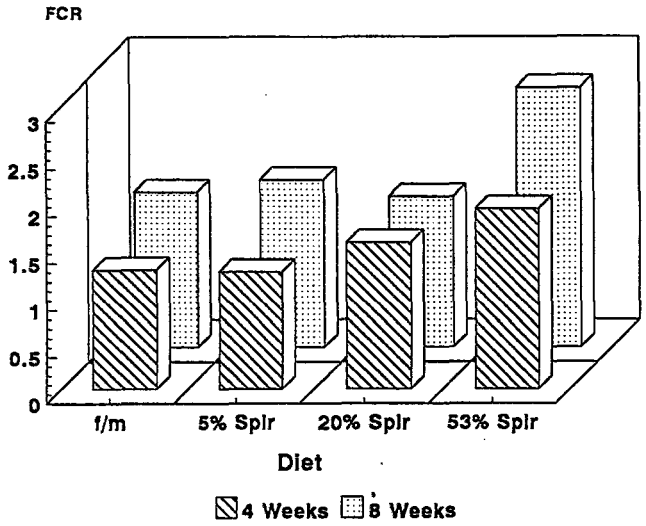

Figure 8.6 Feed conversion ratios of trout fed a range of test diets.

A trend of increasing FCRs with increasing levels of *Spirulina* was apparent. Experimental error due to mortality and the measured feeding regime probably accounts for the variation present. It must be noted, however, that the lower concentrations of *Spirulina* produce FCRs and FERs within the range of that of pure-fishmeal fed animals.

The PERs of the various test diets are shown in Figure 8.7.

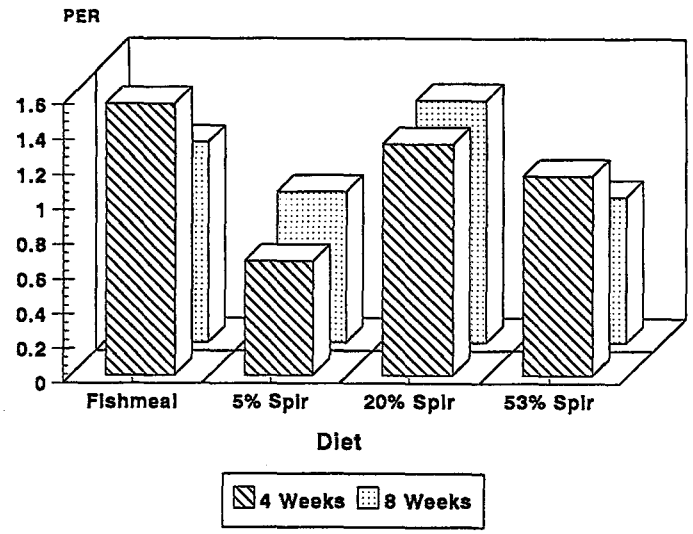

Figure 8.7 Protein efficiency ratios of trout fed a range of test diets.

It was unfeasible to subject these results to statistical analyses because of the small sample size. Each tank of fish was treated as one sample, as the fish could not be marked and monitored individually.

8.4.2 Histopathological Examination

Photographs from the microtome sections of heart, liver, kidney, spleen and intestine are shown in Figures 8.8 - 8.12. Representative samples from the control group (pure-fishmeal) and the 53% *Spirulina* feeding groups are shown.

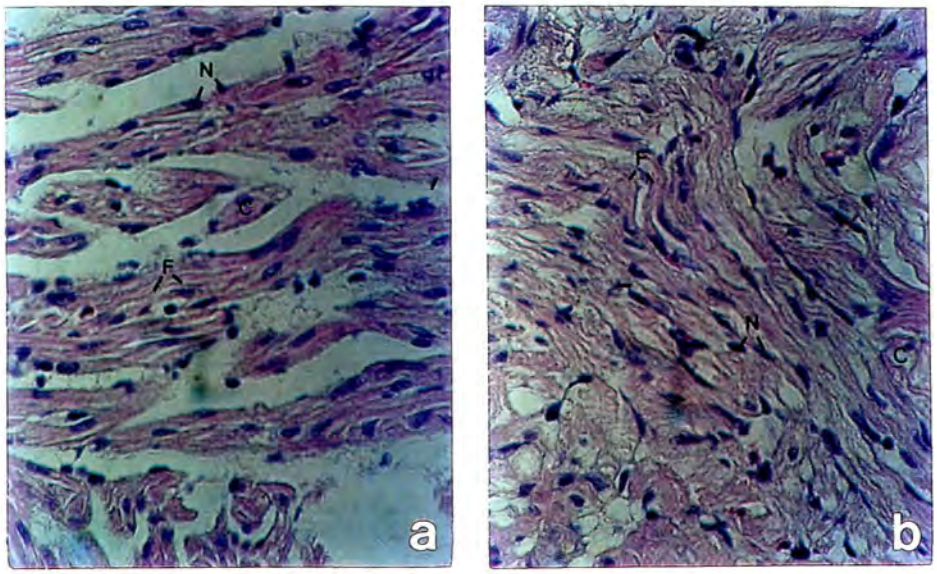

Figure 8.8 Cardiac muscle sections of trout fed pure-fishmeal (A) and 53% *Spirulina* supplemented (B) diets (mag. = 100X): **C** = cross-cut fibres; **N** = nuclei; **DN** = distorted nuclei; **F** = myofilbrils. No specific changes between the cardiac muscle of the various feeding groups can be noticed.

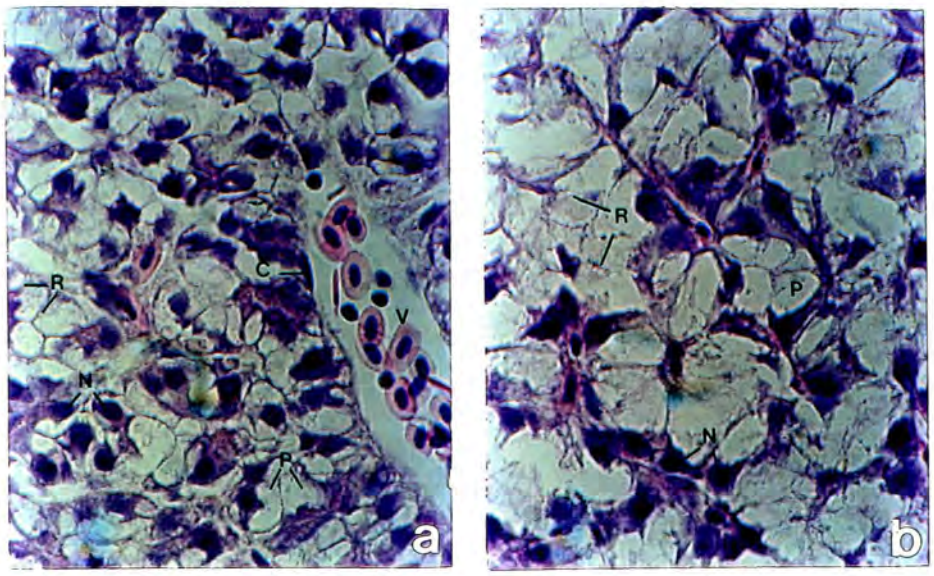

Figure 8.9 Liver sections of trout fed pure-fishmeal (A; mag = 100X) and 53% *Spirulina*-supplemented (B; mag = 160X) diets: **C** = collagen fibres in wall of central vein of lobule; **N** = nuclei of liver cells; **P** = parenchyma cells; **R** = reticular fibres; **V** = central vein of lobule (tributary of the hepatic vein). Note the diffuse fatty hepatosis (indicated by the large vacuoles) in the tissues from all the feeding groups.

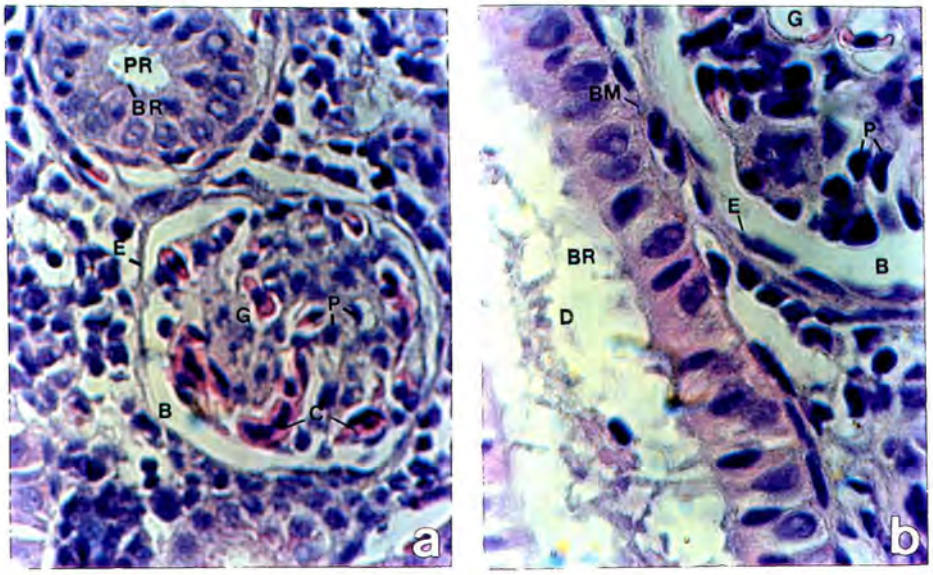

Figure 8.10 Kidney sections of trout fed pure-fishmeal (A; mag = 100X) and 53% *Spirulina*-supplemented (B; mag = 160X) diets: **B** = Bowman's capsule; **BM** = basement membrane; **BR** = brush border; **D** = distal convoluted tubule; **C** = capillaries; **E** = epithelium of capsule; **G** = glomerulus; **P** = podocyte nuclei; **PR** = proximal convoluted tubule. There are no significant morphological lesions in any of the kidneys examined. The only change noted is that some of the tubules from the *Spirulina*-fed trout contain, or are occluded by basophilic lamellated calculi.

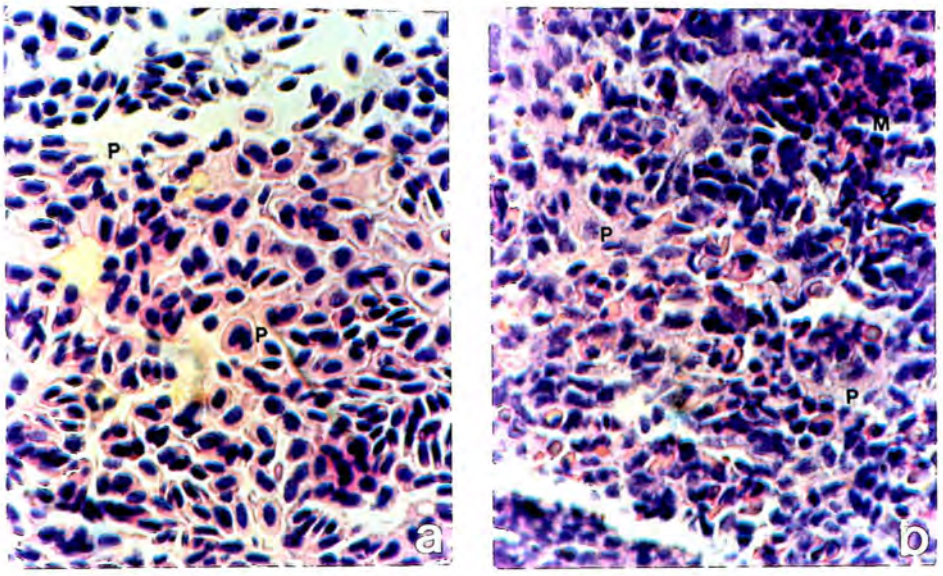

Figure 8.11 Spleen sections of trout fed pure-fishmeal (A) and 53% *Spirulina*-supplemented (B) diets (mag. = 160X): **M** = malpighian corpuscle; **P** = pulp. There are no morphological differences between the spleens from the different feeding groups.

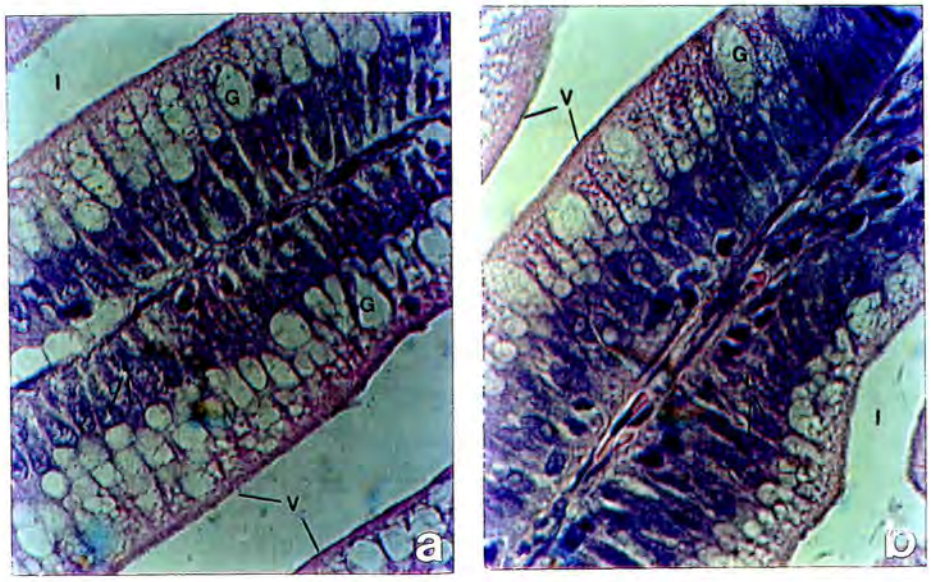

Figure 8.12: Intestine sections of trout fed pure-fishmeal (A) and 53% *Spirulina*-supplemented (B) diets (mag. = 160X): **G** = goblet cell; **I** = intestine lumen; **L** = crypt of Lieberkuhn; **V** = villus. No specific diet-related changes can be noted in the intestines from the different feeding groups.

8.4.3 Pigment Analysis

Total carotenoid analyses of the skin and muscle are shown in Figure 8.13.

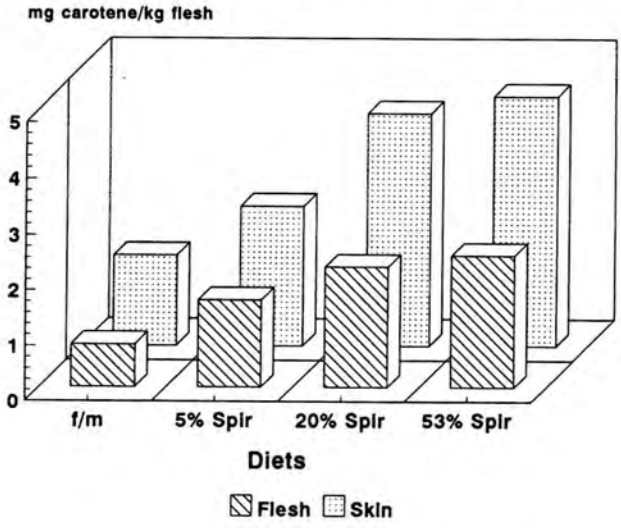

Figure 8.13: Colour analysis of skin and muscle from trout fed a range of *Spirulina* supplemented diets. Each result is the mean of the spectrophotometric analysis of three fish.

There is a concomitant increase in the amount of pigment with a rise in the degree of *Spirulina* supplementation, both in the skin and the muscle.

8.5 DISCUSSION

8.5.1 Mortality

Mortality rates were significantly high during the first week of the feeding trial. It was thought that mortality may be due to a gram negative bacterial infection, probably a mixed infection of *Pseudomonas* and *Aeromonas spp.* (Martin Davies, pers. comm.). Treatment with oxytetracycline, an antibiotic active against gram negative bacteria, resulted in a significant reduction in mortality. Initial mortality is therefore probably due to handling stress during the initial weighing and transfer of the experimental animals. Although the sudden change in diet may have been a contributing factor, mortality is unlikely to have been diet related, as animals from the control feeding groups exhibited similar rates to those of the experimental animals.

8.5.2 Growth Responses

The primary aim of the above series of experiments and analyses was to determine whether tannery effluent-grown *Spirulina* can replace a portion of the normally-used fishmeal in the diets of rainbow trout. The SGRs (Figure 8.4) indicate that lower concentrations of *Spirulina* provide a full spectrum of available amino acids required for growth (reinforced by the amino acid contents shown in Table 8.4), and result in growth rates similar to those obtained with fishmeal. The growth rates exhibited by animals fed higher concentrations of *Spirulina* are lower than the fishmeal-fed animals. The general conclusion of other authors (substantiated by this study) is that lower concentrations of microalgae provide an adequate feed supplement to fishmeal (Ahmad, 1966; Gupta & Ahmad, 1966; Matty & Smith, 1976; Reed *et al.*, 1974; Sandbank & Hepher,1980; Stanley & Jones, 1976). Watanabe *et al.* (1990, cited in Okada *et al.*, 1991) investigated the growth of striped jack, and reported that fish reared on a diet containing 5% *S.maxima* grew very well, but those fed on a 10% supplemented diet did not

exhibit favourable growth. This is reinforced by this study, which indicates that over-supplementation with *Spirulina* is not desirable.

The FCRs (Figure 8.6) also indicate that lower concentrations of *Spirulina* provide an adequate protein supply for the nutritional maintenance of physiological growth, although these values must be treated with a certain amount of caution due to the inherent error associated with measured feeding (Cowey, 1992; Halver, 1989). The most accurate way of feeding is either by manual- or automated-demand feeding (Cowey, 1992), and this is unfeasible considering the number of experimental tanks used and the feeding frequency of fingerling trout.

The most accurate analysis of the feeding trial is that of weight increase, and values obtained in this study indicate that it seems feasible to incorporate lower concentrations of *Spirulina* in the diets of rainbow trout, without any significant decrease in growth rates. The nutritional advantages following the incorporation of effluent-grown *Spirulina* in the diet of rainbow trout have been noted by other authors. Growth rates of animals fed lower concentrations of the biomass are comparable to growth rates of fishmeal-fed animals. *Spirulina* contains most of the essential nutrients, and it is known that addition of *Spirulina* to fish feeds increases the feed palatability (Henson, 1990), indicated by the enhanced feed consumption exhibited by the animals in this study. The enhanced feed conversion efficiency for animals fed a 5% supplement can be explained by an improvement of the intestinal flora, which leads to improved digestion. The intestinal flora induced by feeding with *Spirulina* produce useful vitamins and displace potentially harmful pathogens. Supplementation with *Spirulina* also stimulates production of enzymes that stimulate fat transport, redirecting this storage compound into active growth (Henson, 1990).

8.5.2 Histopathological Examination

The safety of the use of tannery effluent-grown *Spirulina* is reinforced by the histopathological study on the main target organs for toxic compounds. Analysis, and subsequent examination by a veterinary pathologist in Cape Town, South Africa, showed that there were no manifestations of toxicity in the hearts from the various feeding groups. The

cardiac muscle appears unaffected, and the striations appear unchanged from that of the control.

The intestines examined all appear normal. The columnar epithelium lining the mucosa does not manifest any toxicological symptoms, and the brush border of microvilli indicates no absorptive malfunction. The enzyme- and mucus-secreting goblet cells scattered among the epithelial cells do not exhibit histopathological symptoms of toxic contact. The submucosa, and the circular and longitudinal muscles of the muscularis also appear of normal orientation and morphology.

The spleens from all the feeding groups also show no signs of altered morphology. There is thus no malfunction in leucocyte production, indicating that the immune system of the animals have not been challenged by toxic compounds. The Malpighian corpuscles enveloping the smaller arteries are of normal density and frequency, as is the surrounding less-dense red pulp.

The livers of all the feeding groups (including the controls) exhibited diffuse fatty hepatosis indicated by the large vacuoles present in the tissues. This is apparently a common feature of fish farmed commercially (Lucia Lange, pers. comm.), and it is unlikely to have been caused by the diets. Fatty hepatosis was also observed by Kamata *et al.* (1990) in the cultivation of rainbow trout using *A.aestivalis* as a dietary pigment source. This, however, was not diet related, as both the experimental and control groups exhibited the liver abnormality, and substantiate the findings of this study that the fatty deposits are not a direct result of feeding with *Spirulina*. It is also reported that lipids are present in large quantities in fish livers (Fange & Grove, 1979), although no reference to salmonid livers is made. Hydrocarbons are known to accumulate in the liver, aiding in fish buoyancy (Fange & Grove, 1979).

All other cells of the liver appear unaffected. The parenchymatous tissue show no signs of degeneration. The epithelial lamellae separating the blood sinusoids appear unaltered. The sinusoids themselves show typical orientation around the intralobular (central) vein, indicating no spatial, and thereby, functional, abberation from the norm.

The kidney was the only organ examined that seemed to indicate some toxicological manifestation following ingestion of *Spirulina*. The tubules of the kidneys from the groups fed *Spirulina* supplements contained, or were occluded by, basophilic lamellated calculi. This type of tubular aberration may be caused by excessive CO_2 levels in the water (Lucia Lange, pers. comm.), magnesium deficiency (Cowey & Sargent, 1979) or selenium toxicity (Lucia Lange, pers. comm.), and is an early manifestation of nephrocalcinosis (deposition of calcium, leading to stone formation) (Heptinstall, 1966).

It is unlikely that magnesium deficiency is the cause of these early symptoms of nephrocalcinosis, because of the inherent magnesium content of the biomass (Chapter 6), and the mineral supplement in the diets which contains sufficient magnesium to sustain cellular functions. It is also noted by Cowey & Sargent (1979) that metabolic disturbances caused by magnesium deficiency may be counteracted by sufficient calcium in the diet. Both the magnesium and calcium content necessary for cellular maintenance in rainbow trout was catered for by the mineral mixture, according to the levels suggested by Halver (1989), notwithstanding the adequate content of these elements in the *Spirulina* biomass. In fact, it was estimated that the mineral content of certain microalgae is sufficient to fulfil the mineral requirements of a variety of freshwater fish, including rainbow trout (Fabregas & Herrero, 1986).

The possibility exists that selenium toxicity may be the cause of the tubular aberrations, but this cannot be ascertained without determination of the levels of this metal in the biomass and kidney tissue, the quantification of which was hindered by the problems experienced with the standard atomic absorption spectra of this metal. It thus remains to be determined whether the levels of selenium in the *Spirulina* biomass are acceptable from a nutritional and toxicological viewpoint.

Nephrocalcinosis has also been noted in humans with increased alkali intake, leading to hypochloremic alkalosis and dehydration (Heptinstall, 1966). This is of interest because of the high alkalinity of the effluent from which this source of *Spirulina* is harvested. As noted in Chapter 6, the kidneys of the chickens fed a 50% supplement of *Spirulina* exhibited degeneration of individual tubular epithelial cells. It was postulated that this may have been

caused by dehydration due to the high NaCl content of the *Spirulina* biomass. It may thus be that the symptoms of nephrocalcinosis observed in the kidneys of the experimental fish may have been caused by the high NaCl content of the biomass, although this is unlikely as dehydration would not present a problem in aquatic animals.

The final hypothesis as to the cause of the kidney disorder relates to the CO_2 content of the water used in the housing of the experimental animals. The experimental system employed in the feeding experiments uses recirculated water, which feeds from the biological filter, to the header tanks, to all the indoor experimental and indoor and outdoor large-stock holding tanks. The large-stock holding tanks have a very high stocking density, the CO_2 levels being kept down by the continual aeration with medical oxygen. It is thus possible that the elevated CO_2 concentration in the water may have been caused by the high stocking density of some of the tanks in the recirculating system, leading to pathological manifestation in the kidneys (Lucia Lange, pers. comm.).

Fish are well known to possess enhanced detoxification mechanisms as compared to those of land vertebrates. They can rapidly solubilize and excrete toxic compounds (Forster & Goldstein, 1969). It is thus unlikely that the abberations of the kidney are due to toxic organic molecules, as they can be rapidly eliminated through the lipoidal gill epithelium membranes into the external aqueous environment (Forster & Goldstein, 1969). The initial fear that histopathological abberations may have been caused by undetectable amounts of pesticides thus appear to be unfounded, as Buhler (1966, cited in Forster & Goldstein, 1969) demonstrated the inducibility of hepatic drug-metabolising enzymes upon exposure of rainbow trout to the pesticides DDT and phenylbutazone, the end-products of which are excreted by specific tubular transport mechanisms.

With the exception of the kidney tissue abberations, all the other major target organs for toxic compounds do not exhibit any symptoms of toxicosis. Further elucidation is needed to determine unequivocally whether the nephrocalcinosis exhibited by the experimental animals is due entirely to the supplementation of the fishmeal diets with effluent-grown *Spirulina*.

8.5.3 Colour Enhancement

The carotenoids in the skin of *Spirulina*-fed animals seemed to be localised mainly in the pink band along the lateral line, a phenomenon also noted by Storebakken & No (1992). There was a visually apparent enhancement of colour with an increase in the degree of dietary *Spirulina* supplement.

Figure 8.13 shows the colour enhancement potential of *Spirulina* supplementation in rainbow trout. There is an increase in total carotenoid content of both the skin and the flesh with an increase in the degree of supplementation. The intensified colouration of the fish is due to the high pigment concentration in the biomass. This colour enhancement phenomenon following *Spirulina* supplementation in the diets was also noted in sweet smelt (Mori *et al.*, 1987), striped jack (Okada *et al.*, 1991) and Japanese Ayu (Henson, 1990).

Although research into the colouration of table fish centres mainly on the flesh colour, market research has shown that the Japanese consumer places emphasis on both flesh and skin colouration (Arai *et al.*, 1987). Researchers have found extensive deposition in the both flesh and skin of fish (Gentles & Haard, 1991; Kamata *et al.*, 1990), substantiated by the results of this study. All the trout examined for carotenoid content exhibited enhanced deposition in the skin, with the carotenoid concentration in the skin being almost double that of the muscle. This substantiates the observations of other authors. Kamata *et al.* (1990) found a greater total carotenoid deposition in the skin of *Adonis*-fed rainbow trout. Gentles & Haard (1991) also observed this phenomenon when feeding rainbow trout with various extracts of the red yeast, *P.rhodozyma*. Bjerking *et al.* (1990) also found that marine trout accumulate more pigment in the skin than in the flesh, and it is thought that carotenoids are initially stored and metabolised in the subcutaneous tissue, and then transferred to the integument (Mori *et al.*, 1987). It is theorised (Gentles & Haard, 1991; Long & Haard, 1988; Storebakken *et al.*, 1987) that skin pigmentation precedes flesh pigmentation, which may explain the greater carotenoid content of the skin. Although this observation has been reinforced by Hardy *et al.* (1990) in their studies with deposition of C^{14} labelled canthaxanthin, it seems an unlikely explanation for the greater skin deposition observed in the experimental animals in this study.

Results shown in Figure 8.13 indicate that carotenoid deposition in both the skin and muscle increases with a concomitant increase in the carotenoid concentration in the experimental diets, which is not in accordance with the observations of Mori *et al.* (1987), who found that there was no appreciable difference in the colouration of sweet smelt when fed with various supplementations of *Spirulina*. There does not seem to be a cut-off threshold in skin deposition, but it seems likely that deposition occurs simultaneously in the both the flesh and skin. It is possible however that the threshold depends on the dietary pigment concentration, and that the experimental time (8 weeks) was not sufficiently lengthy for the threshold levels to be reached. It may be that the different carotenoids are deposited at different rates, as Miki *et al.* (1985, cited in Okada *et al.*, 1991) reports that tunaxanthin in cultured yellowtail occupy the same proportion of total carotenoids, and is independent of the carotenoid concentration in the diet.

Although there is an increase in both skin and flesh colouration with an increase in carotenoid content, it is unfeasible to incorporate such high concentrations of the biomass in the diet of the rainbow trout, as higher concentrations of *Spirulina* leads to reduced growth rates. Also, a practical long term supplementation of lower concentrations of the alga will lead to sufficient deposition of carotenoids in the skin. The economic advantage of the faster colour enhancement induced by higher *Spirulina* concentrations is overridden by the reduced growth rate, when one considers that the main criterion for table fish quality is mass, the colour being a secondary, albeit economically important, consideration. Extraction of the carotenoids from the biomass is an unfeasible prospect, as the extraction process is expensive. Also, Long & Haard (1988) found that the xanthophyll pigmentation of rainbow trout is more efficient when pigment is added to the ration as carotenoprotein rather than as free carotenoid.

This study thus indicates that it is feasible to incorporate low levels of effluent-grown *Spirulina* in the artificial diets of rainbow trout in order to intensify both skin and flesh colouration, bestowing an economic advantage to fish raised on a *Spirulina*-supplemented diet.

CHAPTER 9

CONCLUSIONS AND RECOMMENDATIONS

The biotechnology of cultured *Spirulina* is a well-developed industry, with commercial plants having been established worldwide (Richmond, 1986b). Research has centred on defined-media culture, and the problems and capital input associated with this type of culture system is the reason for the high selling price of this source of dietary protein. Earthrise Farms in California, for example, markets their source of *Spirulina at* $15.50-19.00/kg.

Natural blooms of *Spirulina* have been noted centuries ago. This study, however, is the first report of an attempt to utilise a natural bloom of *Spirulina* in animal nutrition.

The cascade of evaporation ponds at WTC in which *Spirulina* prevail are characterised by chemical and organic pollutant gradients. The COD levels, which give an indication of the organic pollution level decreases along the cascade, concomitant with an increase in prevalence of *Spirulina*. It has already been established that various species of cyanobacteria are capable of heterotrophic nutrition, and organic uptake by *Spirulina* may partly explain the decrease in organic load along the cascade. If this is the case in this cultivation system, by the definitions of Oswald (1988a), the occurrence of *Spirulina* in tannery effluent represents a naturally-occurring saline facultative lagoon. Theoretically, this effluent-treatment system should perform two tasks: production of maximal algal biomass coupled with wastewater purification. Ideally, the pond should operate without changes in the dominant algal species, as the harvesting technique is tailored for a specific species, and the potential use of the algal species is targeted. The saline and alkaline character of the wastewater inhibits the growth of most microorganisms, and thus bestows a selective advantage for the unchallenged growth of *Spirulina*.

One important criterion for maximal algal growth, that of mixing (Ganapati, 1975), is not satisfied in the effluent ponds. Mixing would produce an overlapping of bacterial oxidation

and photosynthetic reduction, would prevent loss of CO_2 and NH_3 from the bacterial phase, and would provide an effluent outlet for the O_2 liberated by the algae, resulting in more profuse growth of both bacteria and algae.

The large area and great depth of the evaporation ponds (overriding the advantage of temperature stability noted by de Pauw & Persoone, 1988) makes mixing a formidable task, considering the installation and maintenance expense of mixing mechanisms such as propeller or screw pumps, airlift pumps, and rotating brushes. Paddle wheels are relatively cheaper (Oswald, 1988b), with the largest algal pond area mixed by this mechanism being the experimental ponds at the University of California, where two 1000m^2 ponds are stirred by paddles at 12-15cm/s. Recently, efficient Air-O_2 aerators have been developed, which may have application in efficient mixing of the large-volume effluent ponds, for optimisation of *Spirulina* biomass growth (Dave Render, Circuit Engineering, pers. comm.). Again, this would raise the capital outlay, maintenance and power costs, which would need to be weighed against the yield improvement.

Another mechanism that can be employed to enhance *Spirulina* growth in the initial ponds is to install either an aerobic or anaerobic pretreatment pond, which would remove much of the organics hindering cyanobacterial growth in the high-COD initial ponds (Abeliovich, 1986).

The ponding cascade presently supports the growth of 37 tonnes of cyanobacterial biomass, which represents a concentration of 9.5 tonnes.ha^{-1}. It is envisaged that the biomass output can significantly increased if the above considerations are borne in mind.

The most striking aspect of the chemical composition of the effluent-grown *Spirulina* biomass is the relatively low protein and amino acid contents - protein being about 10-20% less than the average of the published values, and many of the essential amino acids being limiting in terms of the levels suggested by the FAO. This could be due to one of four factors:

1. The biomass was harvested by skimming the autoflocculated surface mat. Cell damage, rupture and decomposition may have occurred due to thermal and/or photodynamic death associated with high temperatures and levels of illumination at the

surface. This may have been enhanced by bacterial oxidation, resulting in a leached cell mass being harvested, with subsequent loss of protein and, by definition, amino acids.

2. Protein loss and decomposition may have occurred due to rupturing by the mechanical shear of removal of the dewatered slurry from the screen and transfer to the drying beds.

3. The low protein yield can also be attributed, in part, to the relatively high ash content (15.04% dry weight). This represents the inorganic component of the biomass, contributed, in part by absorbed and adsorbed salts and unused minerals.

4. The fourth, and most likely reason for the low protein yield, may be the lengthy drying time associated with sun-drying. This may allow thermal, followed by bacterial decomposition of the protein.

The following recommendations can thus be made in an attempt to obtain a product with a higher protein content:

1. Harvest of the water column biomass, as apposed to the autoflocculated surface mat. This would ensure harvesting of healthy cells that have not been subjected to thermal and illumination stresses associated with cells in the floating mat. This would, however, necessitate the use of a large-throughput pump, which would increase the harvesting costs in terms of capital outlay and power consumption.

2. Acid-washing of the biomass to remove adsorbed carbonates and other salts. This method was used by Richmond (1988) to reduce the ash content of *Spirulina*. This procedure may increase the protein content/dry weight, when considering the high inorganic salt and mineral content of the biomass.

3. An improved method for rapid, complete dewatering of the cell slurry is needed. A modification of the sun-drying technique is envisaged, as this method employing solar

energy is the most cost-effective for drying of the biomass. A method used by Lincoln & Hill (1980) seems feasible, where the drying method utilises a black cloth surface over an expanded metal mesh with a low UV resistant polyethylene film roof. The slurry is pumped onto the beds, the water filters through, and evaporative drying reduces the final moisture content to 15% or less. Once the cake layer has begun to dry, it cracks and exposes the black cloth beneath it, thus increasing the heat absorbed and speeding the ultimate loss of moisture.

4. A solar heater may also be constructed, as described by Venkataraman *et al.* (1980). This would greatly reduce the drying time. However, due to the large surface area required for the drying of thin cakes, this does not seem to be feasible for large-scale drying.

5. A different fabric, as apposed to the muslin cloth used, could lead to improved drying. Lincoln & Hill (1980) found that a double layer of sailcloth was the most effective and least expensive. Alternatively, muslin cloth could still be used, but prior washing of the new cloth is necessary so as to remove the water-repellant starch which hinders the filtering of the effluent.

Harvesting of the biomass from the effluent is easily achieved due to the relatively large size and helical structure of the trichomes. Both the small- and technical-scale harvests demonstrate the feasibility of screen harvest of the biomass. The simplicity of the technology involved ensures minimal harvesting costs. It is envisaged that the next step towards harvest optimisation would need to involve some automation, with mass pumping of the effluent, followed by screening, and automatic transfer to drying racks. This would minimise harvesting of degraded biomass from the surface flocculated mat, which, as previously noted, may be the cause of the low protein yields observed.

A preliminary evaluation of the harvested biomass showed that the use of effluent-grown *Spirulina* has no detectable toxicological constraints, in terms of heavy metal content, pesticide content, the survival of the sensitive *A.salina* larvae in a bioassay, and the relatively low nucleic acid content as compared to other microbial sources of protein. An intensive

chicken feeding trial also showed that, not only can *Spirulina* replace an economically significant portion of the protein, but there are no unequivocable chemical or pathological manifestations of toxicity in the prime target organs following an intensive feeding trial.

Due to the already stringent control measures on the use of microbial protein in human nutrition, the exploitation of this source of effluent-grown *Spirulina* can only realise its full potential as an animal feed supplement. The preliminary work demonstrating toxicological safety points towards the feasibility of incorporating effluent-grown *Spirulina* as a protein source in aquaculture rations. The next step was then to verify the already existing knowledge on the beneficial properties of *Spirulina* supplements in aquaculture nutrition.

It was demonstrated that this source of *Spirulina* can adequately replace up to 10% of the fishmeal protein in the diet of the South African abalone, *Haliotis midae*. This has implications in the artificial-diet rearing of this high-priced delicacy, the technology of which is currently being developed for adaptation to South African conditions.

Furthermore, it was also demonstrated that tannery effluent-generated *Spirulina* can replace up to 20% of the fishmeal protein in the diet of the rainbow trout, *Oncorhynchus mykiss*. Supplementation with *Spirulina* resulted in growth rates similar to fishmeal-reared animals, with no decisive chemical or pathological manifestations of toxicity. In addition, supplementation with *Spirulina* also results in enhancement of flesh and skin colour, an important consideration of aesthetic quality in the marketing of this table fish.

The specific aim of this study was to demonstrate that tannery-effluent generated *Spirulina* can be feasibly incorporated into the diets of aquaculture organisms. This was by no means an exhaustive study, as the use of *Spirulina* in aquaculture supplements has already been well documented. An attempt was thus made at identifying potential problem areas associated with effluent-grown biomass. The feasibility of incorporation from a nutritional and toxicological viewpoint has been demonstrated, although further feeding trials with a range of aquaculture organisms need to be conducted.

From an economic viewpoint, however, the exploitation of effluent-generated *Spirulina* as a protein source can only be fully realised if the cost benefits associated with *Spirulina* supplements override those of the economics of the use of fishmeal. Diet formulation studies need to be conducted, with "least-cost programming" presently being used by feed manufacturers (Peter Britz, pers. comm). These programmes evaluate formulated feed in terms of nutritional content and cost of ingredients. Thus, based on its nutritional profile, a cost threshold will exist beyond which the incorporation of the ingredient will not be cost effective. It was noted by Hacking (1986) that, in general, the bulk products of biotechnology show high elasticity of demand in response to price, as they are challenged by other products or methods of production. Microbial sources of protein is one of the products which exhibits the highest elasticity, as it is competing with other products, notably soybean and fishmeal, for which it can be directly substituted.

There is a noticeable lack of information available on the costing analysis of microalgal systems. This can be almost entirely ascribed to the industrial secrecy that prevails around microalgal production, and reference to costs incurred only being dealt with superficially by academic scientists. Benneman *et al.* (1987) provides one of the few general costing analyses of microalgal production. Becker & Venkataraman (1980) also noted that production costs of pure-culture grown biomass are too high to become economical, and the advantages associated with easy harvest and processing are annulled by these high production costs. By extrapolation of information from Hacking (1986), the selling price of pure-culture grown *Spirulina* is in the same range as that of fishmeal.

As noted, however, costs associated with exploitation of this source of *Spirulina* are minimal compared to pure-culture grown biomass. An algal production system would incur major costs associated with pond construction, inoculum system, harvesting, media and process control. Because the technology of pond construction and media supply are already accounted for in terms of the effluent-treatment cascade and influent wastewater, production costs of this effluent source of *Spirulina* would essentially only involve the financial aspects of harvesting. Berend *et al.* (1980) estimate harvesting costs at 10% of the total production costs, which is within the range found by Benneman *et al.* (1987), who estimate costs at 4.5-13% of the total operational costs.

Costs associated with drying would also be minimal, due to the relatively low technology involved in sun-drying.

It is noted by Hacking (1986) that various factors affect the selling price of a product. Aside from the effective costs associated with production, the product destination determines the final selling price. If this source of effluent-grown *Spirulina* is destined as a protein source in aquaculture rations, the price must be competitive to that of fishmeal, which currently retails at approximately R 1 500.tonne^{-1} (Peter Britz, pers. comm). Another option exists in the marketing of *Spirulina* as a specialist aquaculture supplement, most noteworthy as a colour enhancement supplement in already existing protein rations. If this were to be implemented, the retail price would far exceed that obtained with marketing of the product as a protein source alone.

Despite the lack of hard costing data on the effluent-production of *Spirulina*, it seems that there is great potential for the development of this source of protein to prosper into a biotechnological industry, if not as a replacement for the fishmeal component in aquaculture diets, then as a specialist feed for colour enhancement. A product of value is thus obtained with a concomitant decrease and removal of nutrients from the effluent in terms of biomass. The pollutant load is reduced, ultimately allowing for discharge into the public water system.

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ACKNOWLEDGEMENTS

My sincere thanks to the two Peters, Rose and Britz, for their guidance and enthusiasm, persevering through disasters of escaped chickens, half-a-thousand carp belly-up, and insurmountable times when a swift kick to my rear would not have been uncalled for. Thanks especially to the first Peter for introducing me to the fields of environmental and commercial algal biotechnology, and for lessons that often extended beyond the academic.

And, in no particular order, my gratitude and indebtedness to:

Moira Pogrund, for loud, efficient technical service, without which the department would be in a flurry.

Kevin "Yo" Dunn, for updating of the data on the *Spirulina* standing crop, and in conjunction with Oleg Shipin for execution of the technical-scale harvest.

Roger Rowswell (Leather Industries Research Institute, Grahamstown) for design of the screen harvester, and LIRI technical staff for assistance with chemical tests.

Martin Davies (Department of Ichthyology and Fisheries Science, Rhodes University) for very enthusiastic assistance with the trout feeding trial.

Bruce Fivaz, Charles Willemse and Eddie Williams for help with the chicken feeding trial.

The staff at Western Tanning Company, Wellington.

Lesley Horne, for carotenoid and xanthophyll determinations.

Dr Lucia Lange for assistance with the histopathological study.

South African Bureau of Standards for pesticide evaluation.

Department of Animal and Poultry Science, University of Natal, for amino acid analysis.

Financial assistance from the Foundation for Research and Development, and the Water Research Commission is also acknowledged.

My deepest gratitude also to my parents for a chance at a great education, and the magnificent people both in and out of the department who made everyday living mostly fun.

APPENDIX I

The investigations detailed above have been presented as follows:

1. **The Growth and Production of *Spirulina* in Tannery Effluent.**
   South African Society for Microbiology (SASM) Seventh Biennial Congress, March/April 1992, Bloemfontein.

2. **Toxicological and Nutritional Evaluation of Effluent-Grown *Spirulina*.**
   South African Society for Microbiology (SASM) Seventh Biennial Congress, March/April 1992, Bloemfontein.

3. **Application of Effluent-Grown Halophilic Algae in the aquaculture Production of Fish and Shell-Fish.**
   Marine, Estuarine and Freshwater Ecosystems Conference, July 1992, Rhodes University, Grahamstown.

4. **Use of Effluent-Grown *Spirulina* as an Aquaculture Ration.** Aquaculture '92 Congress, September 1992, Rhodes University, Grahamstown.

APPENDIX II

The following projects have arisen as a result of the above investigations:

1. Biological treatment of saline organic tannery effluent using *Spirulina* in a High Rate Oxidation Ponding system.

2. The removal of heavy metals from industrial effluent by *Spirulina*.

3. The effect of chemical and environmental conditions on the essential fatty acid content of *Spirulina*.

4. Anaerobic digestion of solid tannery waste, using *Spirulina* as a carbon source.

5. Use of effluent-grown *Spirulina* for colour enhancement of ornamental fish.

6. An industrial-scale evaluation of the use of effluent-grown *Spirulina* as a feed source in a variety of aquaculture organisms.